Abstracts

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Oral Contributions

(ordered according to sessions)

Molecular Mechanisms Controlling Leukocyte Extravasation

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The entry of leukocytes into sites of injury or infection requires molecular mechanisms that enable the leukocyte to recognize such sites from within the vasculature and to form contact to the endothelium in order to exit and migrate through the blood vessel wall. Recognition as well as contact formation is mediated by several cell adhesion molecules acting in a sequential manner in concert with regulatory mediators such as the chemokines. The cell adhesion molecules involved in this process belong to three gene families: the selectins, the integrins and the immunoglobulin super gene family. The importance of the leukocyte integrins and the selectins is highlighted by two human genetic defects, the leukocyte adhesion deficiency (LAD) I and II. Whereas the genetic defect-causing LAD I is known since several years the molecular nature of the defect causing LAD II has only recently been revealed and will be discussed. In contrast to the well-analyzed process of leukocyte association with the blood vessel wall the process of leukocyte migration across the endothelial cell layer is not yet well understood. Indirect evidence suggests that the docking of leukocytes to the apical surface of endothelial cells triggers the opening of endothelial junctions. Endothelial membrane proteins likely to be involved in the control of endothelial junction integrity and/or leukocyte extravasation are VE-cadherin, PECAM-1 and JAM. JAM is the prototype of a small family of JAM-related proteins that seem to be associated with tight junctions and that may represent interesting candidates for regulators of cell contacts. Beside such tight junction associated components, the major mediator of endothelial cell contact stability is VE-cadherin. A novel mechanism will be discussed that is able to influence VE-cadherin-mediated cell contact integrity.

Encephalitogenic T Cells Use LFA-1 for Transendothelial Migration but Not during Capture and Adhesion in Spinal Cord Microvessels in vivo

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LFA-1 on the surface of encephalitogenic T cells has been suggested to be involved in the pathogenesis of experimental autoimmune encephalomyelitis (EAE). By applying a novel technique of intravital fluorescence microscopy that allows us to visualize the interaction of circulating encephalitogenic T lymphoblasts within the healthy spinal cord white matter microvasculature in vivo, we investigated the possible involvement of LFA-1 on circulating encephalitogenic T cells in their multi-step interaction with the blood-brain barrier (BBB) endothelium in vivo. LFA-1 was found to neither mediate the G-protein independent capture nor in the G-protein dependent

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adhesion strengthening of encephalitogenic T cell blasts within spinal cord microvessel. In contrast, blocking LFA-1 on encephalitogenic T lymphoblasts resulted in a significantly reduced number of T cells migrating across the vascular wall into the spinal cord parenchyme. Our study provides the first direct in vivo evidence that encephalitogenic T cells use LFA-1 for transendothelial migration but not for capture and adhesion in spinal cord microvessels in vivo.

Slow E-Selectin Mediated Leukocyte Rolling Is Dependent on Sialyltransferase ST3Gal-IV

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Leukocyte rolling is an early event in the recruitment of leukocytes into sites of inflammation and is mediated by selectins and their counter-receptors called selectin ligands. Selectin ligands carry core2 decorated, fucosylated and sialylated O-glycans, which are crucial for binding to selectins. At least six sialyltransferases (ST3Gal I–VI) have been identified that may contribute to selectin ligand formation. Using intravital microscopy we investigated E- and P-selectin-mediated leukocyte rolling in a model of acute inflammation (2–4 h TNF-α) in cremaster muscle venules of mice deficient in ST3Gal-IV. Injection of blocking mAbs against either P-selectin or E-selectin caused changes in the number of rolling leukocytes that paralleled those in control mice suggesting normal leukocyte rolling in ST3Gal-IV$^{-/-}$ mice. However, analysis of leukocyte rolling velocities for E-selectin mediated rolling (after P-selectin blockade) revealed a significantly higher average rolling velocity (9 ± 0.4 μm/s) in ST3Gal-IV-deficient mice than in control mice (6 ± 0.4 μm/s). Our data indicate that ST3Gal-IV is required for the synthesis of selectin ligand(s) supporting E-selectin-dependent rolling at the characteristically slow rolling velocities, but is not involved in regulating P-selectin dependent rolling or initial attachment (capture) on E-selectin.

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The Thy-1/Thy-1 Ligand Interaction Is Involved in Binding of Melanoma Cells to Activated Thy-1-Positive Microvascular Endothelial Cells

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Cell adhesion plays a central role in metastasis of tumor cells as well as inflammation. Cell adhesion mediated by specific adhesion molecules direct and regulate the invasion of cells in certain tissues. In recent studies, we characterized the human Thy-1 molecule as an inducible activation-associated cell adhesion molecule on human dermal microvascular endothelial cells (HDMEC). Thy-1 expression has never been detected on resting endothelial cells in situ and in vitro. The Thy-1 ligand was detected on granulocytes and monocytes. Our experiments proved the involvement of the Thy-1/Thy-1 ligand interaction in binding of PNC and monocytes to activated Thy-1-positive HDMEC. The strong connection of inflammatory events and Thy-1 expression suggests a specific role during binding of monocytes and PNC to activated endothelium establishing an event-specific interaction with PNC and monocytes from peripheral blood vessels during inflammatory responses or cell activation processes. Furthermore, we could demonstrate a strong expression of Thy-1 on endothelial cells in tissue section of melanoma. In vitro, an induction of Thy-1 expression on HDMEC was seen after stimulation with melanoma cell derived soluble mediators. The Thy-1 ligand was found on different melanoma cell lines in vitro and on melanoma cells in situ. In cell adhesion assays the involvement of the Thy-1/Thy-1 ligand interaction in binding of melanoma cells to activated Thy-1-positive HDMEC was shown. Taken together, the investigation of Thy-1 and Thy-1 ligand expression might allow a more detailed insight in the direction and regulation of the invasion of cells in tissue with inflammation as well as in metastasis of certain tumor cells.

Transcriptome Analysis Reveals a Role of Interferon-γ in Human Neointima Formation

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The long-term success of stent-supported angioplasty is limited by restenosis due to neointima formation. To gain further insight into transcriptional and signaling events governing neointima formation, we investigated the expression of 2,435 genes of known function in atherectomy specimens and blood cells of 10 patients with in-stent restenosis (n = 10) as well as in normal coronary artery specimens (n = 11), and cultured human smooth muscle cells (SMCs). While the expression of housekeeping genes was largely comparable between normal and neointimal tissue, an impressive number of genes (n = 223) showed an increased or decreased level of expression. The gene expression pattern in neointima showed the anticipated proliferative response with induction of genes mainly expressed in G1/S phase, changes of the smooth muscle phenotype and changes in synthesis of ECM. Additionally, we observed a pro-inflammatory expression pattern characterized by the presence of markers for macrophages and T lymphocytes and by expression of numerous genes involved in the cellular response to IFN-γ. The IRF-1 protein, a pivotal transcription factor in IFN-γ signaling, was also found overexpressed at the protein level in SMCs of human neointima. Of the 37 genes composing the IFN-γ-associated cluster, 25 (68%) were inducible in cultured SMCs by treatment with IFN-γ. We found that IFN-γ reduced the basal rate of apoptosis in proliferating SMCs and attenuated hydrogen peroxide (H_2O_2)-induced apoptosis significantly. Finally, we demonstrated that mice lacking the IFN-γ receptor had a significantly reduced vascular proliferative response in a model of restenosis. Therefore, we conclude that, similar to atherosclerosis, IFN-γ plays an important role in the control of tissue proliferation during neointima formation.

Mechanisms for Leukocyte-Induced Derangement of the Endothelial Barrier in Acute Inflammation

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Activation and recruitment of polymorphonuclear leukocytes (PMN) is pivotal in the host defense against noxious stimuli; yet a key element in the pathogenesis of inflammatory disease. As a consequence of PMN trafficking, the barrier function of the endothelial lining is adjusted, leading to increased vascular permeability and plasma fluid efflux. Although known to be dependent on the function of leukocytic β_2 integrins (CD11/CD18), the precise mechanisms by which activated PMN may impair endothelial barrier capacity have remained largely unclear. The kinetics of PMN-induced alteration in junctional permeability of endothelial cell (EC) monolayers was investigated together with analysis of EC intracellular activation events. We show that PMN activation, either by chemoattractant stimulation or through antibody cross-linking of CD11/CD18 (mimicking adhesion-dependent receptor engagement) provokes an active response in adjacent EC, manifested as a rise in cytosolic free Ca^{2+} and rearrangement of actin filaments, concomitant with an increase in protein permeability of the monolayer. Our findings demonstrate that engagement of β_2 integrins and transmembrane signaling triggers the secretion of neutrophil-borne cationic proteins that can be held responsible for mediating the PMN-evoked alteration in EC permeability. Heparin-binding protein (HBP/CAP37), an inactive serine protease homologue belonging to the serprocidin family of granule proteins, is critical in this response. HBP stimulates activation of the contact system leading to liberation of bradykinin, a potent inducer of increased vascular permeability. Collectively, our data provide evidence for a paracrine signaling pathway involving contact system activation in the regulation of vascular permeability to plasma proteins and fluid efflux in conjunction with leukocyte trafficking in inflammation.

Involvement of JAM-1 as a Newly Identified Ligand of LFA-1 in Leukocyte Transendothelial Migration

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Inflammatory recruitment of leukocytes is governed by dynamic interactions between integrins and endothelial immunoglobulin superfamily (IgSF) proteins. We have identified the IgSF member junctional adhesion molecule 1 (JAM-1) as a ligand of the beta(2) integrin lymphocyte function-associated antigen 1 (LFA-1). Under static and physiological flow conditions, JAM-1 contributed to LFA-1-dependent transendothelial migration of T cells and neutrophils as well as LFA-1-mediated arrest of T cells. The latter was triggered by chemokines on endothelium that was stimulated with cytokines to redistribute JAM-1 from the tight junctions. Transfectants expressing JAM-1 supported LFA-1-mediated adhesion of leukocytes, which required the membrane-proximal Ig-like domain 2 of JAM-1. Thus, JAM-1 is a counter-receptor for LFA-1 that is ideally situated to guide and control transmigration during leukocyte recruitment. The role of homotypic and heterotypic JAM-1 interactions in a model proposing a multi-layered molecular zipper for leukocyte diapedesis will be discussed.

Functional Impact of Coupling between Leukocytes and Endothelium via Gap Junctions

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Neutrophilic granulocytes (PMN) are able to couple with endothelial cells via gap junctions in a rapid, adhesion-dependent fashion. The functional relevance of this potential communication pathway was, however, unknown to date. We examined effects of cellular coupling on on transmigration of PMN through HUVEC monolayers towards a chemotactic gradient (FMLP). The specific role of gap junctions in this context was investigated by using a peptide (SRPTEK), which blocks the subunits of gap junctions (connexins), and by comparing the behaviour of PMN on HeLa wildtype cells (WT, no connexins) with that of PMN on HeLa cells selectively expressing connexin 40 (Cx40). Transmigration of PMN through confluent monolayers of Cx40 cells (transwell-assay) was 45% lower than through WT, while an ED_{50} of SRPTEK increased transmigration through HUVECs by 21% vs. controls (n = 9 in all groups). Permeability of cell monolayers for fluorescent labelled dextrane increased only marginally during transmigration of PMN in the presence of functional connexins, while in the absence of connexins, permeability was dramatically elevated. Thus, in the absence of connexins (WT) or during functional blockade (SRPTEK), transmigration of PMN is facilitated, as compared to situations, where coupling can occur. This effect of gap junctional coupling could be due to inhibitory signals passed from endothelial cells to PMN. Furthermore, permeability during transmigration is modulated by gap junctional coupling. Bearing in mind that inflammatory mediators reduce gap junctional coupling, this novel way of intercellular communication might prove to be functionally important for discriminating physiological and pathological extravasation.

Kinetic Analysis of Angiogenesis in Human Tumors

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Angiogenesis is now widely recognized as one of the most important tumor progression parameters. Yet, little is known about the nature and the functional status of the angiogenic vascular bed in human tumors. Microvessel density (MVD) counting techniques have become the gold standard to assess the vascular bed in human tumors. However, MVD counts provide a static readout of the tumor

vascular bed, but do not reflect angiogenesis. We have performed an extensive meta-analysis of all published MVD counting studies. This analysis confirmed the limited use of MVD counts as tumor prognostic parameter. Yet, it proved extremely useful to quantitatively assess the architecture of the vascular bed in human tumors, which varies significantly in different type of human tumors. In order to assess quantitative parameters of angiogenesis in human tumors, we have employed histology-based diagnostic angiogenesis techniques. Assessing angiogenesis on the basis of endothelial cell (EC) proliferation revealed significant differences in the intensity of angiogenesis between different tumor types (glioblastomas > renal cell carcinomas > colon carcinomas > mammary carcinomas > lung carcinomas > prostate carcinomas) as well as a marked heterogeneity within individual groups of tumors. When relating the intensity of angiogenesis to individual patient's prognosis (recurrence, 5-year survival), close to 80% of mammary carcinoma patients with EC proliferation indices of >10% had a poor prognosis confirming the role of active angiogenesis for tumor progression. Taken together, the data support the concept that active angiogenesis is one of the most critical positive tumor progression factors. Yet, implementation of quantitative angiogenesis diagnostic techniques is presently one of the critical rate-limiting steps in translating the promising results of ongoing angiogenesis research therapeutically into the clinic.

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The Cytochrome P450 2C9 Epoxygenase Induces Angiogenesis via Cross-Talk with the Epidermal Growth Factor Receptor (EGFR)

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Cytochrome P450 (CYP) epoxygenase products, such as 11,12-epoxyeicosatrienoic acid (EET), exert a proliferative effect on endothelial cells. We set out to identify the signal transduction cascade linking EET generation to enhanced proliferation and to determine whether or not EETs are angiogenic factors. In human endothelial cells over-expressing CYP 2C9, cell number as well as bromodeoxyuridine incorporation into DNA were increased compared to control vector treated cells. Both effects were sensitive to the selective CYP 2C9 inhibitor sulfaphenazole. CYP 2C9 over-expression was associated with the activation of Akt and an increase in cyclin D1 expression, effects which were abolished by AG1478, a specific inhibitor for the EGFR tyrosine kinase. AG1478 also prevented the CYP 2C9-induced increase in cell proliferation. CYP 2C9 over-expression or stimulation with 11,12-EET enhanced EGFR tyrosine phosphorylation in EGFR-over-expression cells, indicating that both endogenously generated as well as exogenously applied EETs activate the EGFR. Since activation of the EGFR with EGF induces angiogenesis, we determined whether the over-expression of CYP 2C9 also induces angiogenesis. Human endothelial cells were seeded on a fibrin gel and infected with adenoviral constructs encoding CYP 2C9 sense or antisense or treated with 11,12-EET. CYP 2C9 over-expression as well as 11,12-EET significantly enhanced tube formation. Also in an in vivo angiogenesis assay, 11,12-EET induced vessel formation and convergence. These results indicate that CYP 2C9-derived EETs stimulate endothelial cell proliferation and angiogenesis. The signalling pathway involves the activation of Akt and cyclin D1 upregulation and is initiated by activation of the EGFR.

Wall Shear Stress Is Essentially Involved in Angioadaptation

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The development and maintenance of the vascular system requires not only the formation of new vessels (vasculogenesis, angiogenesis) but also the continuous adjustment of vessel and network structures in response to functional needs. This 'angioadaptation' depends on the interplay of vascular responses to growth factors, to the metabolic status of the tissue, and to hemodynamic forces exerted by the flowing blood. To determine the role of wall shear stress in angioadaptation, a mathematical model to simulate microvascular adaptation has been developed. In addition, the microcirculation was studied in several animal models by means of intravital microscopy, and the influence of wall shear stress on endothelial gene expression in vitro was investigated. In vitro, using a cone and plate system to expose endothelial cells to laminar flow (6 dyn/cm^2, 24 h), expression of tie-2, METH-1, and gas-3 was strongly induced, while ang-2, TSP-1, and CD36 were inhibited. This corresponds with data from microvascular networks. Highest shear stress values are seen in short segments with high PO_2 levels, whereas lowest shear stress values are found in longer segments with low PO_2 levels. Thus, balanced flow distribution prevents an overall disparity in oxygen supply. For this purpose, structural responses, i.e. angioadaptation, especially to wall shear stress, is required. Flow-regulated gene expression may be a key mechanism underlying these responses.

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Shear Stress-Dependent Regulation of Mediators of Vascular Development and Remodeling Ephrin-B2 and Angiopoietin-2 in Human Endothelial Cells

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Endothelial phenotype and development of atherosclerosis might involve local differences in hemodynamic forces. Ephrin and angiopoietin receptor tyrosine kinases play an essential role in vascular development and remodeling. In this study, primary cultures of human umbilical vein and artery endothelial cells (HUVEC,

HUAEC) were exposed to laminar shear stress (1, 5, 10, 15, 30, 50 dyn/cm^2 for up to 24 h) in a cone-and-plate viscometer. Ephrin-B2 expression is downregulated in a dose-dependent manner by arterial laminar shear stress on gelatine-coated plates in HUVEC (max. at 30 dyn/cm^2, 24 h: 46 ± 4% of internal control without shear stress, n = 16, $p < 0.05$). In contrast, venous levels of shear stress did not affect ephrin-B2 expression. Downregulation of ephrin-B2 by arterial shear stress is prevented by protein kinase C inhibitor RO 31-8220 (1 μ*M*) and on collagen I- and laminin-coated plates. In contrast, ephrin-B2 is not downregulated by arterial shear stress in HUAEC. ANGPT-2 is 1.6-fold upregulated by venous levels of shear stress (1 dyn/cm^2, 24 h, n = 10), but downregulated to 57 ± 10% by arterial shear stress (max. 30 dyn/cm^2, 24 h, n = 16) on gelatine-coated plates. A similar regulation of ANGPT-2 can be found on collagen I and laminin. Downregulation of ANGPT-2 involves PKC. In HUAEC no upregulation by venous, but downregulation of ANGPT-2 by arterial shear stress could be found. Shear stress-dependent downregulation could be mediated by reduced binding of transcription factor AP-1 to ephrin-B2 or ANGPT-2 promoter (max. 30 dyn/cm^2, 24 h). In summary, we show a downregulation of ephrin-B2 and ANGPT-2 by chronic arterial shear stress in HUVEC. Signal transduction pathway involves PKC and cell-matrix-interactions. This might contribute to vascular integrity in response to laminar shear stress.

VEGF Receptor Signalling and Endothelial Function

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Vascular endothelial growth factor has initially been described as an endothelial-specific growth factor and inducer of angiogenesis. More recently, a number of extra-endothelial activities have been demonstrated for VEGF and VEGF-receptors have been identified on a wide range of cell types. Nevertheless, the effect of VEGF on endothelial cells and monocytes seems to be the most prominent one in health and disease. VEGF stimulates both the differentiation of endothelial cells (VEGFR-2 is an early marker on haemangioblasts) as well as activity of endothelial cells including nitric oxide production and release. It has recently been realized that the endothelium needs a constant stimulation with VEGF as reflected by detectable tissue and serum level of VEGF. The inhibition of VEGF signalling using specific VEGF-receptor inhibitors leads to endothelial cell apoptosis and tissue destruction. VEGF activates the vascular endothelium via two different receptor tyrosine kinases denominated VEGFR-1 and VEGFR-2. It was recently shown, that the activity of VEGFR-2 is controlled by the activity of VEGFR-1. The activation of VEGFR-1 contributes to activation of VEGFR-2 (Biological Transistor Model). VEGF cannot only trigger angiogenesis, but also arteriogenesis. This growth of preformed collaterals (arterioles) into large arteries can be achieved by activation of the endothelium as well as by recruiting and activating monocytes into the growing arterial wall. The enhancement of arteriogenesis by VEGF can result in enhanced tissue perfusion. The activation of monocytes as well as the activation of endothelial cells is impaired in monocytes derived from diabetic patients. Diabetes mellitus is associated with signal transduction defects in both monocytes and in endothelial cells with immediate consequences for monocyte and endothelial function. These findings establish VEGF as a central regulator of vascular function.

Hypoxic/Ischemic Induction of the Wilms' Tumor Transcription Factor Wt1 in the Coronary Vasculature

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The coronary vasculature is formed by epicardium-derived cells that undergo mesenchymal transition during cardiac development. The Wilms' tumor gene *Wt1* encodes a transcription factor, which has been implicated in the reciprocal transition of cells between an epithelial and mesenchymal state. Among other organ defects, *Wt1*-deficient mouse embryos exhibit extreme thinning of the myocardium. Disturbed cardiac growth in the *Wt1* null mutants is presumably due to disruption of the epicardial layer, which is the only site of *Wt1* expression in wild-type hearts. Here we demonstrate a more than 3-fold increase of *Wt1* mRNA in the left ventricles of rats with myocardial infarction. *Wt1* expression was elevated between 1 day and 9 weeks after coronary artery occlusion. Remarkably, in situ mRNA hybridization and immunohistochemistry revealed *de novo* expression of *Wt1* in the coronary vasculature in the border zone of the infarcted tissue. Vascular co-localization of *Wt1* and proliferating cell nuclear antigen (PCNA) as well as vascular endothelial growth factor (VEGF) was demonstrated by double-immunofluorescent labeling of tissue sections from infarcted rat hearts. Up-regulation of *Wt1* in the vasculature of ischemic hearts could be mimicked by exposure of rats to normobaric hypoxia (8% O_2) and 0.1% carbon monoxide, respectively. *Wt1* was co-localized with hypoxia-inducible factor HIF-1α in the coronary vessels of hypoxic hearts. In conclusion, induction of the Wilms' tumor transcription factor in proliferating coronary vascular cells after myocardial infarction suggests a role for *Wt1* in coronary vascular formation in the ischemic heart. It is suggested that Wt1 enables the formation of new blood vessels in the heart by allowing cells in the myocardial vasculature to acquire a mesenchymal phenotype.

Redox Regulation of HIF-1α and HIF-2α Expression in Pulmonary Aortic Smooth Muscle Cells

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Objectives: Hypoxia-inducible factor-1α (HIF-1α) and HIF-2α/endothelial PAS domain protein (EPAS) are basic-helix-loop-helix (bHLH)-containing members of the PER-ARNT-SIM (PAS) family of transcription factors that play a central role in the response to hypoxia. Recently, non-hypoxic regulation of HIF-1α has also been reported. However, the underlying mechanisms are not understood.

We therefore investigated, whether reactive oxygen species (ROS) are involved in hypoxic and non-hypoxic HIF regulation. **Results:** Human pulmonary aortic smooth muscle cells (PASMC) were stimulated with thrombin, the hypoxia mimetic $CoCl_2$ or exposed to hypoxia (1%) and Northern and Western blot analyses were performed. All three stimuli increased HIF-1α and HIF-2α expression at the protein and at the mRNA level. Pretreatment with the antioxidants vitamin C (100 μM), N-acetyl cysteine (10 mM) and pyrrolidine dithiocarbamate (100 μM) abrogated upregulation of HIF-1α and HIF-2α protein and mRNA levels. Moreover, exposure to H_2O_2 increased HIF-1α and HIF-2α protein expression in a dose-dependent manner. In contrast, treatment of the cell line ECV304 or human umbilical vein endothelial cells with H_2O_2 did not modulate HIF expression. ROS measurements using the DCF technique further indicated increased generation of ROS in response to thrombin and $CoCl_2$. **Conclusions:** ROS inhibit the upregulation of HIF-1α and HIF-2α in response to hypoxic and non-hypoxic stimulation in a cell type-specific manner in PASMC.

Shear Stress-Induced BFGF Release from Endothelial Cells Is Dependent on Integrin and Matrix Protease Activity

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Vascular remodeling following chronically elevation of shear stress (SS) has similarities with the response to bFGF suggesting a mediator role of this factor. However, up to date little is known whether and by which mechanism SS induces bFGF release. We studied whether SS elicits bFGF release from endothelial cells and whether specific cell matrix interactions are involved. Endothelial cells (EC) were kept under static conditions (SC) or subjected to SS (20 dyn/cm^2). Conditioned media from SS or SC experiments were tested for bFGF (ELISA or proliferation-differentiation assay). In part of the experiments, inhibitory peptides of cell matrix interactions as well as the protease inhibitor aprotinin were added during the SS period. During 6h SS, bFGF release was 10-fold higher than in SC ($p < 0.05$, n = 6). LDH concentration was similar in both, SS and SC. In bioassays, only SS-conditioned medium induced proliferation ($p < 0.05$, n = 6) and capillary-like structure formation ($p < 0.05$, n = 6). Either neutralising antibodies to bFGF or inhibitory peptides against its receptor abolished both effects whereas antibodies against VEGF had no effects. Release of bFGF (ELISA) was abolished completely by incubation with RGDSP (inhibiting αvβ3 integrins; n = 12) but neither by GRDNP (inhibiting α5β1) nor YIGSR (inhibiting LBP). When SS experiments were performed under inhibition of elastase with aprotinin, we also found no shear induced bFGF release. Moreover, after 2 h of incubation with elastase alone, bFGF release increased to a similar amount as under SS (n = 4). Western blots showed that the bFGF after protease treatment was indeed released from EC and not proteolytically from the matrix. The results indicate that shear stress is a potent stimulus for the release of fully active bFGF that may act as mediator of its proliferative effects. The mechano-sensing and/or release mechanism is critically dependent on matrix modulation via elastase and specific cell matrix interactions via αvβ3 integrins.

Role of the Plasminogen System for Collateral Artery Growth

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To define the role of the plasminogen activators (PAs) urokinase PA (uPA) and tissue PA (tPA) as well as the uPA receptor (uPAR) in arteriogenesis, we investigated their impact in a rabbit and mouse model of adaptive collateral artery growth. Collateral artery growth was induced by occlusion of the femoral artery in rabbit and wild-type (WT) mice and in mice with targeted inactivation of uPA ($uPA^{-/-}$), tPA ($tPA^{-/-}$), uPAR ($uPAR^{-/-}$). Northern blot results revealed a significant upregulation of uPA but not uPAR or tPA in the early phase of arteriogenesis in rabbit and WT mice. This upregulation on RNA level was followed by an increased protein level and enzymatic activity. Impaired perfusion recovery upon femoral artery ligation was observed by laser Doppler analysis in vivo in uPA deficient mice but not in uPAR or tPA deficiency compared to WT mice. Immunohistochemical studies revealed an association of leukocyte infiltration with arteriogenesis in WT mice which was strongly reduced in $uPA^{-/-}$ but not in uPAR or tPA deficient mice. From our study we conclude that arteriogenesis is promoted by a uPA mediated infiltration of leukocytes which is not dependent on uPAR.

Embryonal EPCs Attenuate Postischemic Reperfusion Injury in Mice Hearts

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Recent reports have demonstrated a beneficial role of adult endothelial progenitor cells (EPCs) in the setting of myocardial ischemia and reperfusion. EPCs from bone marrow or circulating blood, expanded ex vivo and infused after the ischemic insult, contributed to reduced loss of myocardial tissue and improved function after a subacute remodeling interval (2–4 weeks). Since a sufficient number of adult EPCs is not always available for application rapidly after an acute ischemic insult, we investigated the potential of embryonal EPCs to antagonize ischemia/reperfusion injury in mice. Moreover, we investigated mechanisms of PMN and EPC recruitment, since polymorphonuclear granulocytes contribute to endothelial and myocardial dysfunction after ischemia and reperfusion and blockade of PMN adhesion might interfere with EPC recruitment. **Methods:** Mice (n = 6 per group, wildtype or ICAM-$1^{-/-}$) were anesthetized and subjected to LAD ligation for 20 min. With the onset of reperfusion, 2×10^5 Di-I labeled EPCs were infused into the external jugular vein for 15 min. Thereafter, hearts were arrested and analyzed by fluorescence microscopy for cell recruitment. In parallel experiments (n =

6), circulating leukocytes were stained with rhodamine-6-G and leukocyte adhesion was studied after 15 min of reperfusion. In a chronic model of ischemia (1 h) and reperfusion (14 days) we investigated the influence of EPC and PMN recruitment on systolic myocardial function (left ventricular developed pressure = LVDP) and development of heart failure (LV end-diastolic pressure = LVEDP). **Results:** Compared to non-ischemic control hearts, ischemia and reperfusion (I/R) induced a significant increase in both, EPC recruitment (10.8 ± 1.8 vs. 1.5 ± 0.4/field) and PMN adhesion (55 ± 5 vs. 8 ± 2/field). ICAM-1 deficiency, however, reduced PMN recruitment (17 ± 2/field) and not EPC recruitment (8.8 ± 0.8/field). In the chronic model, I/R induced a significant loss of systolic function (LVDP 81 ± 5 vs 112 ± 4 mm Hg in sham-operated animals), which was attenuated by EPC infusion (2×10^5 cells 24 h after ischemia, LVDP 95 ± 4 mm Hg). Interestingly, ICAM-1 deficient mice also displayed an improvement of LVDP (93 ± 4 mm Hg), which was further extended after EPC-infusion (102 ± 8 mm Hg). LVEDP increased after I/R (7.3 ± 1.1 mm Hg vs. 4.2 ± 0.3 mm Hg). Either EPC-infusion or ICAM-1 deficiency did not change the elevation of LVEDP (6.8 ± 1.3 mm Hg and 7.1 ± 1.5 mm Hg, respectively). Infusion of EPCs in ICAM-1 deficient mice, however, reduced LVEDP to 4.3 ± 0.8 mm Hg. We conclude that postischemic EPC recruitment does not depend on endothelial ICAM-1 expression, in contrast to postischemic PMN adhesion. Moreover, ICAM-1 absence and EPC infusion exert additive cardioprotective effects in a chronic model of ischemia and reperfusion. Whether the effect of EPCs relies on induction of postischemic angiogenesis of the ischemic area, remains to be determined.

Adventitial $VEGF_{165}$ Gene Transfer Inhibits Constrictive Remodeling and Luminal Loss after Porcine Coronary Angioplasty

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Experimental studies have shown that vascular endothelial growth factor (VEGF) mediates non-angiogenic vascular protective mechanism and reduces neointima formation after arterial injury. The effect of VEGF on vessel geometry after injury is unknown. The purpose of this study was to examine the effect of (peri)adventitial $VEGF_{165}$ gene transfer after coronary balloon injury on 1) vascular remodeling and 2) lumen area (LA). Porcine coronary arteries were examined on days 3, 7, 14 and 28 after arterial injury and either adventitial $VEGF_{165}$ or LacZ gene transfer. Coronary segments were analyzed using immunohistochemistry and image analysis. LA increased in the control (LacZ) group until day 3 followed by continuous lumen loss between 7 and 14 days after injury. In the VEGF group no lumen loss was observed and this was due to an increase in the external elastic lamina area (EELA; positive remodeling). Positive remodeling in the VEGF-treated group was associated with increased adventitial neovascularisation in comparison to the LacZ group (VEGF vs. LacZ: * $p < 0.05$).

	Mean area, mm²				
	injury	day 3	day 7	day 14	day 28
LacZ LA	2.94±0.1	4.63±0.4	3.29±0.3	2.35±0.1	2.47±0.2
VEGF LA	2.94±0.1	4.26±0.9	4.35±0.5	3.77±0.3*	4.19±0.7*
LacZ EELA	3.67±0.2	6.01±0.6	5.01±0.3	4.65±0.3	5.48±0.4
VEGF EELA	3.67±0.2	5.39±1.0	6.02±0.5	5.91±0.2*	7.56±0.6*

Local (peri)adventitial $VEGF_{165}$ gene transfer after coronary artery angioplasty suppresses constrictive remodeling and inhibits late lumen loss. Positive remodeling may be due to VEGF-induced adventitial neovascularisation, possibly by inhibiting arterial wall hypoxia and fibrosis.

Influence of Cryopreservation on Angiogenesis of Freely Transplanted Ovarian Follicles

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Introduction: The technique of cryopreservation and autologous transplantation of ovarian tissue is a promising approach to restore fertility function of female cancer patients suffering from iatrogenic infertility due to chemo- or radiation therapy. However, little is known about the influence of the freeze-thaw procedure on the process of ovarian angiogenesis. Therefore we transplanted ovarian follicles, which had been cryopreserved, into dorsal skinfold chambers of Syrian golden hamsters and analyzed the graft's potential to induce revascularization using intravital fluorescence microscopy. **Methods:** Ovarian follicles were isolated from PMSG-stimulated hamsters and transplanted into dorsal skinfold chambers of synchronized animals either after 2 weeks of cryostorage or directly after isolation (control). Neovascularization of the grafts was analyzed over 10 days after transplantation by assessment of revascularized follicular tissue area, microvessel density, microvessel diameters and hemodynamics, leukocyte-endothelial cell interaction and macromolecular leakage using in vivo fluorescence microscopy. **Results:** All cryopreserved and nonpreserved follicles showed adequate vascularization after transplantation. First signs of angiogenesis, i.e. capillary sprouts and sinusoidal sacculations, could be observed at day 3. At days 7–10, grafts exhibited complete glomerulum-like microvascular networks with a microvessel density of ~ 300 cm/cm^2 and a revascularized area of 92 ± 4% in cryopreserved and 109 ± 10% in control follicles. Diameters of the grafts' capillaries were found reduced from 14–16 μm at day 3 to 10–11 μm at day 10. Calculated values of volumetric blood flow ranged between 10 and 20 pl/s throughout the observation period. Significant differences between the two groups, however, could not be observed. Enhanced leukocyte-endothelial cell interaction was absent in follicles upon revascularization. **Conclusion:** Cryopreservation does not affect neovascularization of freely transplanted ovarian follicles.

Dephosphorylation of the Angiotensin-Converting Enzyme (ACE) Determines Its Cleavage-Secretion from Endothelial Cells

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Soluble Angiotensin-converting enzyme (sACE, 175 kDa) is derived from the membrane-bound form (pmACE, 180 kDa) of the enzyme by proteolytic cleavage of the C-terminal domain. Since the intracellular phosphorylation of proteins can regulate cleavage processes, we determined whether the phosphorylation of the cytoplasmic tail of ACE regulates its secretion. Immunoprecipitation of the pmACE from ^{32}P-labelled human endothelial cells revealed that ACE is indeed phosphorylated. Phosphorylation was absent in endothelial cells over-expressing a mutant form of ACE (ACEΔS, all cytoplasmic serine residues replaced by alanine). The cell supernatant from ACEΔS cells contained substantial amounts of sACE whereas pmACE was hardly detectable in these cells, although a precursor ACE (170 kDa) was prominent in the endoplasmic reticulum. In endothelial cells over-expressing wild-type ACE, the CK2-inhibitor 5,6-dichloro-1-(beta-D-ribofuranosyl) time-dependently decreased the phosphorylation of ACE and increased its secretion. Furthermore, CK2 was co-precipitated with ACE from endothelial cells and in in vitro kinase assays the CK2 phosphorylated ACE as well as a peptide corresponding to its cytoplasmic tail. Selective point mutation revealed that ACE is predominantly phosphorylated on Ser1270, which is situated within a consensus sequence for the CK2. Secretion of the Ser1270Ala mutant was significantly greater than that of wild-type ACE under control conditions and was unaffected by the CK2 inhibitor. These results demonstrate that ACE is phosphorylated by CK2 in endothelial cells, a process which regulates the retention of ACE in the plasma membrane and as a consequence endothelial cell-associated ACE activity.

Selective Blockade of Endothelial Ca^{2+}-Activated Small- and Intermediate-Conductance K^+ Channels Suppresses EDHF-Mediated Vasodilation

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Activation of Ca^{2+}-activated K^+ channels (K_{Ca}) has been suggested to play a key role in endothelium-derived hyperpolarizing factor (EDHF)-mediated vasodilation. However, it is still elusive which K_{Ca} mediate cell hyperpolarization and thus EDHF-mediated vasodilation. Using the new non-cytochrome P450 blocking clotrimazole-derivatives, TRAM-34 and TRAM-39 as highly selective inhibitors of the intermediate-conductance K_{Ca} (IK1), we investigated the functional role rat IK1 (rIK1) in endothelial hyperpolarization and EDHF-mediated vasodilation. Expression and function of the rIK1and the small-conductance K_{Ca} (rSK3) were detected in situ in single endothelial cells of rat carotid arteries (CA) by whole-cell patch-clamp measurements and single-cell RT-PCR. rIK1 currents were blocked by charybdotoxin (ChTX), clotrimazole (CLT), TRAM-34, or by TRAM-39, while rSK3 was blocked by apamin. In current-clamp experiments, endothelial hyperpolarization in response to acetylcholine was abolished by the combination of apamin and TRAM-34. In phenylephrine-preconstricted CA, acetylcholine-induced NO and prostacyclin-independent vasodilation was almost completely blocked by ChTX, CLT, TRAM-34, or TRAM-39 in combination with the SK3 blocker apamin. Apamin and clotrimazole alone or sulphaphenzole, a blocker of the cytochrome P450 isoform 2C9, were ineffective in blocking the EDHF response. In experiments without blocking NO^- and prostacyclin synthesis, the combined blockade of SK3 and IK1 reduced endothelium-dependent vasodilation by ~25%. In conclusion, activation of both K_{Ca}, rIK1 and rSK3 in the endothelium of rat CA is crucial in mediating endothelial hyperpolarization and generation of the EDHF signal. In contrast, arachidonic acid metabolites of the cytochrome P450 pathway seem to play a minor or no role in these vessels.

Voltage-Gated Calcium Channels in Renal Resistance Vessels: Implications for Regulation of Renal Blood Flow

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There exists solid functional and pharmacological evidence for the presence of distinct, segment-specific calcium influx pathways in renal microvessels; however the molecular correlates for this reactivity and the sites of their expression has remained elusive. We have explored the distribution of voltage-dependent calcium channels in kidney resistance vessels at the molecular and functional levels. RT-PCR analysis of microdissected rat preglomerular vessels, juxtamedullary efferent arterioles, descending vasa recta, and cultured renal smooth muscle cells showed co-expression of mRNAs for T-type subunits ($Ca_V3.1$, $Ca_V3.2$), for an L-type subunit ($Ca_V1.2$) and for the P/Q-type subunit ($Ca_V2.1$). No calcium channel messages were detected in cortical efferent arterioles. $Ca_V1.3$, $Ca_V2.3$ and $Ca_V3.3$ were not detected in renal vessels. Immunochemistry showed that $Ca_V1.2$ and $Ca_V2.1$ were associated with smooth muscle cells of rat preglomerular vasculature, juxtamedullary efferent arterioles and vasa recta. Cortical efferent arterioles were not immunopositive. Recordings of intracellular calcium concentration with digital fluorescence imaging microscopy showed a significant increase of calcium in response to K^+ (100 mmol/l) in isolated afferent arterioles (140 ± 25%) and in juxtamedullary efferent arterioles (118 ± 21%). These calcium responses were attenuated by the L-type antagonist calciseptine, by the P/Q-type antagonist ω-agatoxinIVA and by the T-type antagonist mibefradil. Each of the antagonists mibefradil, nickel, calciceptine and ω-agatoxinIVA concentration-dependently and reversibly blocked K^+-induced contraction of perfused rabbit afferent arterioles. We conclude that voltage-dependent P/Q-, L- and T-type calcium channels are expressed in a segment-specific fashion in the renal microvasculature and that several calcium channels contribute to K^+-induced contraction in renal afferent arterioles and in juxtamedullary efferent arterioles.

TRPC Calcium Channels of Vascular Smooth Muscle: Store Refilling, Contraction and Other Functions

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The contractile phenotype of vascular smooth muscle cells is clearly associated with the expression and function of L-type voltage-gated calcium channels. However, in addition, these cells also express other – non-voltage-gated – calcium channels, about which we know very little. In the *Drosophila* eye there is a calcium channel protein which in mutant form leads to a transient receptor potential (TRP) in response to bright light. At least 19 TRP-related human gene products have recently been discovered. Members of the TRPC subfamily of these proteins are particularly associated with store- and receptor-operated calcium channel functions, which also exist in vascular smooth muscle. We have shown TRPC1 is expressed as mRNA and protein in a range of vascular smooth muscle types, including in terminal arterioles of pial microcirculation [Xu and Beech 2001, Circ. Res. 88, 84–87]. It occurs in the plasma membrane and a functional – blocking – TRPC1-specific antibody partially inhibits store-operated calcium entry in arteriolar smooth muscle cells. But TRPC1 may not act alone. We detect other TRPCs at mRNA and protein levels and show TRPC5 co-immunoprecipitates with TRPC1 in medial layer of human saphenous vein. The proteins both localise to arteriolar smooth muscle cells. Strikingly, store-operated calcium entry in arterioles does not cause contraction, despite the global intracellular calcium elevation having the same amplitude as that evoked by 34-m*M* potassium [Flemming et al 2002, J. Physiol; in press]. On this and other evidence we can strengthen the hypothesis that store-operated calcium entry is compartmentalised, conferring on TRPC1 discrete cellular functions including store refilling and perhaps modulation of phenotype.

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Vasomotion in Small Arteries Is Synchronized by a cGMP-Dependent, Calcium-Activated Chloride Current

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In many tissues, small arteries both in vivo and in vitro display regular or irregular variations in diameter or tone (vasomotion) over time that are not dependent on externally generated rhythms. Such coordinated movements of a blood vessel obviously require some means of communication between cells. We have recently [Peng et al., Circ. Res. 88: 810, 2001] hypothesized that virtually all vascular smooth muscle cells are capable of generating oscillations, and that the individual oscillators are synchronized by an electrical signal, generated by intracellular calcium activating a depolarizing ion channel that is cGMP dependent. We have here characterized this channel using the whole-cell patch clamp technique on freshly isolated smooth muscle cells from rat mesenteric small arteries. Elevating intracellular calcium levels by either caffeine, ionomycin, elevated calcium in the holding pipette, or by photolysis of caged calcium all activated an inward current (168 ± 28 pA, n = 20) in the presence of intracellular cGMP or extracellular 8Br-cGMP. The current was not seen in the absence of cGMP, was not sensitive to substitution of either Na^+, K^+, or Ca^{2+}, but its reversal potential followed the Cl^- gradient. It was insensitive to K^+ and Ca^{2+} channel antagonists, but exhibited a moderate sensitivity to Cl^- channel inhibitors. Interestingly, the current was inhibited by Ni^{2+} and Zn^{2+} in micromolar concentrations, but was unaffected by Co^{2+}. The PKG inhibitor, Rp-8Br-PET-cGMP, blocked the current with an EC_{50} of 37 µ*M*. A high concentration (100 µ*M*) of cAMP mimicked the effect of cGMP, an effect that was inhibited by the PKG inhibitor. We thus have characterized what seems to be a novel chloride channel in vascular smooth muscle. Our results are consistent with release of intracellular calcium leading to activation of this cGMP-dependent channel, which conducts a depolarizing current and which may be responsible for the synchronization of oscillations in individual smooth muscle cells and thereby causing vasomotion.

On the Fine Tuning of Arterial Tone by Calcium-Activated Potassium Channels and Calcium Sparks

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Ca^{2+} sparks are thought to represent 'elementary' Ca^{2+} release events, which arise from ryanodine receptor (RyR) channels in the sarcoplasmic reticulum (SR). In arterial smooth muscle, Ca^{2+} sparks have been suggested to oppose myogenic vasoconstriction by activating BK channels (STOCs), consisting of pore-forming alpha and accessory BKβ1 channel subunits. In this paper, I review recent data from our laboratory on the complex relationships of Ca^{2+} influx and Ca^{2+} release within local, subcellular Ca^{2+} microdomains in arterial smooth muscle. A comparative analysis of Ca^{2+} sparks and STOCs in –/– RyR3 and –/– BKβ1 mice is performed. Using intact pressurized arteries, it is investigated whether RyR3 and BKβ1, through membrane potential and *global* cytoplasmic [Ca^{2+}], regulate arterial myogenic tone. Furthermore, the appearance of Ca^{2+} sparks/STOCs in human coronary arteries and the association of single-nucleotide polymophisms in the KCNM1 gene coding for the human BKβ1 subunit with human baroreflex and blood pressure regulation is explored. The results suggest that (1) RyR3 is essential for the release of Ca^{2+} sparks at low physiological frequency, (2) BKβ1 represents the molecular sensor for Ca^{2+} sparks. (3) The Ca^{2+} spark/STOC pathway is required to specifically tune arterial myogenic tone. (4) Mice missing BKβ1 do not sense Ca^{2+} sparks in small arteries and have elevated systemic blood pressure with a reset baroreflex. (5) Variants in vascular channel genes may be responsible for the great range in heart rate variability and baroreflex function observed in humans. Such

variation may also play a role in the development of hypertension. (6) Ca^{2+} sparks and BKβ1 subunits may represent novel potent therapeutic targets for regulating function of intact arterial smooth muscle tissue, including in humans.

NO Donor Sodium Nitroprusside Dilates Rat Small Arteries by Activation of Inward Rectifier Potassium Channels

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The role of vascular smooth muscle inward rectifier potassium (K_{IR}) channels in vasodilations is still unknown. The hypothesis that K_{IR} channels are involved in the sodium nitroprusside (SNP)-induced vasodilation was tested. A pressure myograph, the patch-clamp and the RT-PCR technique and immunocytochemistry were used to determine rat small artery contractile reactions, ion currents and their molecular correlates, respectively. SNP relaxed tail small arteries with an EC_{50} of 2.6 ± 10^{-8} mol/l. Endothelium removal did not attenuate this effect. Vessel pretreatment with 10^{-3} mol/l hydroxocobalamin, an NO scavenger, abolished the SNP effect. Vessel pretreatment with 10^{-5} mol/l barium, a specific blocker of K_{IR} channels at micromolar concentrations, reduced the SNP effect. Low concentrations of potassium dilated the vessels; this effect was attenuated largely after pretreatment with 3 ± 10^{-5} mol/l barium. In freshly isolated smooth muscle cells a barium-sensitive current was observed at potentials negative to the potassium equilibrium potential. Application of 10^{-6} mol/l SNP increased the barium-sensitive current 1.74 ± 0.21-fold at –100 mV. In tissue from freshly dissected vessels transcripts for K_{IR} 2.1 and 2.2, but not for K_{IR} 2.3 and 2.4 were found. However, only K_{IR} 2.1 antibodies immuno-stained the tunica media of the vessel. These data suggest that vascular smooth muscle K_{IR} 2.1 channels are involved in the SNP-induced dilation of rat tail small arteries.

Role of β_3-Endonexin in the Regulation of NF-κB-Dependent Expression of Urokinase-Type Plasminogen Activator Receptor

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Endothelial cell migration during angiogenesis involves adhesive processes mediated by integrins and proteolysis. Previous studies showed that β_3-integrins regulate expression of the urokinase-type plasminogen activator receptor (uPAR) through outside in signalling involving the cytoplasmic domain. We show herein that overexpression of the integrin-binding protein β_3-endonexin decreased uPAR promoter (–398-bp fragment) activity that is constitutively active in endothelial cells. Mutation of the NF-κB promoter binding site (–45-bp) impaired the ability of β_3-endonexin to downregulate uPAR promoter activity. Immunoprecipitation studies showed that β_3-endonexin interacts directly with the p50/p65 transactivation complex and inhibits thereby binding of κB oligonucleotides to the p50/p65 complex. Moreover, binding of β_3-endonexin to p50 was inhibited in the presence of κB but not mutated κB oligonucleotides suggesting a sterically competition between β_3-endonexin and κB DNA for the p50/p65 complex. We therefore propose that β_3-endonexin acts as regulator of uPAR expression in β_3-integrin-mediated endothelial cell migration through direct interaction with p50/p65. Since NF-κB regulates the expression of matrix degrading enzymes the present results define a role of β_3-endonexin in regulating β_3-integrin-mediated adhesion and pericellular proteolysis.

Collateral Artery Growth Is Linked to an Increased Expression of Carp

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Arteriogenesis, the growth of preexisting collateral arteries, can be induced in rabbits by occlusion of the femoral artery. This study aimed to identify genes that are upregulated at early timepoints of the arteriogenic process. The collateral arteries of rabbits 24 h after femoral artery occlusion or sham operation were isolated, the mRNA extracted and amplified by means of the SMART technique. The resulting cDNAs were subjected to suppression subtractive hybridization. In the subsequecent screening steps for truly differentially expressed genes (Slot blot experiments, DNA microarrays), we identified the cardiac ankyrin repeat protein (carp) mRNA to be upregulated. Carp is a nuclear protein that plays a role in the regulation of gene transcription. The increase of the carp mRNA in collateral arteries after femoral artery occlusion was confirmed not only after 24 h and 3 days of experimental procedure, but already 6 h and 12 h after surgery by Northern blot hybridization and quantitative Real Time PCR. Western blot experiments showed that the carp protein was upregulated about twofold in nuclear extracts of collaterals 24 h after occlusion compared to the sham-operated controls. By immunohistochemistry we found carp localized in endothelial cells as well as in smooth muscle cells of collateral arteries. To investigate the influence of substances – known to play a role in arteriogenesis – on the carp mRNA expression in vivo, MCP-1 and TGF-β were infused via osmotic minipumps for 24 h in rabbits. Northern blot experiments showed that MCP-1 had no effect on the carp mRNA level, however, TGF-β was capable of inducing the carp mRNA to about factor 8 of the value of sham-operated animals. The overexpression of the carp protein in Cos-1 cells resulted in an upregulation of the early growth response factor egr-1 as shown by Western blot. In summary, our data demonstrated an association of an enhanced carp expression with the inititation and regulation of collateral artery growth.

Latent Antithrombin (AT) Inhibits Angiogenesis in A-Mel-3-Fortner Melanoma by Delay of Capillary Maturation

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Background: Previous in vitro and in vivo studies have indicated potent antiangiogenic and antitumor activity of cleaved and latent antithrombin (AT). With the use of intravital microscopy, the aim of the present study was to further elucidate the inhibitory action of latent AT on the different steps of angiogenesis, i.e. capillary sprouting, maturation and network formation, in melanoma tumor growth. **Methods:** For these experiments, a skinfold chamber was prepared in 18 Syrian hamsters, and 2×10^5 A-Mel-3-Fortner melanoma cells were implanted onto the skin muscle surface. The process of tumor angiogenesis was analyzed at days 3, 5, 7 and 10 after implantation using intravital microscopy and computer-assisted image analysis. The analysis included the determination of the size of the microvascular network (in relation to the size of the tumor area), microvessel density as well as capillary diameters, red blood cell velocity and blood perfusion. Animals received 25 mg/kg $\times$ d (s.c.) latent (n = 6) or native AT (n = 6). Non-treated animals served as controls. **Results:** During initial angiogenesis, latent and native AT-treated tumors showed significantly larger capillary diameters compared to controls (day 5: 9.5 ± 0.5 µm and 10.2 ± 1.5 µm vs. 7.4 ± 0.5 µm), indicating a delay in capillary maturation. In contrast, neither latent nor native AT affected microvessel density, reflecting a lack of effect on capillary sprouting and vessel formation. However, analysis of the overall size of the microvascular network indicated a significant reduction at day 10 in latent (81 ± 11%) and native AT-treated tumors (88 ± 5%) compared to non-treated controls (143 ± 14%). **Conclusion:** Latent AT does not inhibit capillary sprouting per se, however, may reduce overall tumor angiogenesis by delaying the maturation of the newly formed capillaries. The comparable effectiveness of native versus latent AT is probably due to the fact that native AT contains substantial amounts of spontaneously formed latent AT.

Critical Role of Platelet Adhesion in the Initiation of Atherosclerotic Lesion Formation

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Platelets play a crucial role in thrombembolic complications of advanced atherosclerotic lesions. However, the contribution of platelets to the process of atherosclerosis itself remains unclear. The aim of the present study, therefore, was to evaluate the possible involvement of platelets in atherosclerotic lesion formation. Using *ApoE*-deficient (*ApoE*$^{-/-}$) mice we quantitatively assessed platelet adhesion in atherogenesis. *ApoE*$^{-/-}$ mice were fed a western-type diet for 2, 4, 6, 8, 12 or 18 weeks. Subsequently, platelet-vessel wall interactions were monitored in vivo within the A. carotis com. using intravital videofluorescence microscopy. Platelets were found to adhere to the vascular endothelium of the carotid artery in *ApoE*$^{-/-}$ mice prior to the development of manifest atherosclerotic lesions. Platelet-endothelial cell interaction involved both platelet glycoprotein (GP)Ibα and GPIIb-IIIa. Platelet adhesion to the endothelium coincided with inflammatory gene expression and preceded atherosclerotic plaque invasion by leukocytes. Prolonged blockade of platelet adhesion in *ApoE*$^{-/-}$ mice profoundly reduced leukocyte accumulation in the arterial intima and attenuated atherosclerotic lesion formation in the carotid artery bifurcation, the aortic sinus, and the coronary arteries. These findings establish the platelet as a major player in initiation of the atherogenetic process.

CD18 Gene Dose Controls CD4$^+$ T Cell Emigration Critically Involved in the Psoriasiform Skin Disease in CD18 Hypomorphic PL/J Mice

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CD18 is the common chain of β_2 integrins, which are responsible for adhesion in a variety of inflammatory interactions. In a CD18 hypomorphic PL/J mouse model the severe reduction of CD18 (β_2-integrin) expression to 10% of wild-type mice results in the development of a psoriasiform skin disease. To investigate the role of T cells in the pathogenesis of this dermatitis, immunostainings and in vivo depletion were performed. CD4$^+$ and CD8$^+$ T cells were significantly increased in the skin of affected CD18 hypomorphic compared to wild-type mice. However, only depletion of CD4$^+$ T cells, but not of CD8$^+$ T cells, resulted in a complete resolution of the severe psoriasiform dermatitis, indicating a central role of CD4$^+$ T cells, independent of CD8$^+$ T cells, in the pathogenesis of the disease. In contrast to the CD18 hypomorphic mice, CD18 null mutants of the same strain did not develop the psoriasiform dermatitis. This may be due to a lack of T cell migration out of blood vessels, as after oxazalone challenge, contact hypersensitivity concomitant with a T cellular infiltrate could be induced in CD18 hypomorphic and wild-type mice but not in CD18 null mutants, though primed T cells from the latter were able to cause an inflammatory response when injected directly into subcutaneous tissue of naive syngeneic mice. Hence, 10% of CD18 gene expression is obviously sufficient for T cell emigration from blood vessels driving the psoriasiform phenotype in CD18 hypomorphic PL/J mice. Thus, our data suggest that the pathogenic involvement of CD4$^+$ T cells depends on a gene dose effect with a reduced expression of the CD18 protein in the underlying mouse model.

Magnetofection of Endothelial Cells

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Transfection of plasmidic gene vectors (pDNA) or antisense oligonucleotides (ODN) are attractive tools for investigating cellular signaling. We coupled pDNA or ODN to magnetic micro-particles and transfected them through application of a magnetic field (magnetofection; MF). MF of HUVEC with a luciferase (LF) plasmid elicited an up to 218-fold higher LF activity when compared to 6 different transfection reagents (Effectene EF, PEI, FuGENE FG, DMRIE-C, Lipofectamine, GenePorter, n = 6 each). GFP-transfection efficiency reached 38.7% in HUVEC (n = 6) similar as transfection of a β-gal vector in porcine aortic endothelial cells (37.5%, n = 6). Five minutes of MF of an ODN against the NAD(P)H oxidase subunit $p22^{phox}$ (70 n*M*) reached 80% uptake after 5 min at a toxicity of 4% of cells (n = 8), compared to 77% uptake at a toxicity of 22% after 24 h using the same reagent without MF (n = 8). A faster uptake (15 min) with less toxic effects was also seen for an ODN against cytosolic src-homology 2-containing protein tyrosine phosphatase 1 (SHP-1, 1.5 μ*M*) when using EF, PEI, DOTAP-Chol. or PEI (up to 98%) but not FG (n = 6 each). The ODN transfection was functionally effective since 15 min of MF of the $p22^{phox}$ ODN resulted in a complete loss of phorbol-ester stimulated endothelial O_2^- production 24 h later (n = 6). Likewise expression of SHP-1 protein was inhibited (n = 3), which could not be achieved by conventional transfection. Magnetofection is a novel, faster, more efficient and less toxic transfection technique for endothelial cell cultures.

The Role of Neutrophil-Derived HBP in Monocyte Recruitment to Inflammed Endothelium

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Monocyte infiltration into inflammatory sites is often preceded by neutrophils. However, the mechanisms underlying this second wave of inflammatory response are not fully understood. Recently it has been shown that upon activation and adhesion neutrophils release heparin-binding protein (HBP) [Gautam et al., 2000]. As HBP is known to be a specific chemotactic for monocytes [Pareira et al. 1990, Chertov et al. 1997] we investigated the possible role of neutrophil-derived HBP in monocyte recruitment to inflammed endothelium. Activation of neutrophils, induced by CD18 cross-linking, triggered the secretion and binding of HBP to the cell surface of bovine aortic endothelial cells (BAEC), which was detected by immunofluorescence microscopy. Monocyte arrest on endothelial monolayers or on plates coated with adhesion molecules was studied in a parallel-wall flow chamber assay. We show that immobilised HBP augmented shear resistant monocyte arrest to endothelial cells and to immobilised recombinant adhesion proteins (P-selectin, E-selectin and VCAM-1). Increased binding was found to be specific for monocytes, as HBP did not influence adhesion of neutrophils or lymphocytes. Mobilisation of intracellular free Ca^{2+} in monocytes after HBP stimulation indicated that increased binding is due to activation of the monocytes. We conclude that the deposition of HBP by activated neutrophils on inflammed endothelial cells mediates shear-resistant monocyte arrest. This effect may be important for the second wave of monocyte invasion in inflammation.

Role of RhoA Signalling Pathway in the Control of Smooth Muscle Functions – Regulation by the Nitric Oxide/cGMP-Dependent Protein Kinase Signalling

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The small G protein RhoA and cGMP/cGMP-dependent protein kinase (cGK) pathways exert major and opposing effects on vascular smooth muscle functions such as contraction and growth. We previously demonstrated that interaction between these signalling pathways exists and that cGK phosphorylates RhoA in vitro at Ser 188, leading to an inhibition of RhoA/Rho kinase-mediated Ca^{2+} sensitization of contraction. In addition to this regulation of RhoA-dependent contraction, cGK signalling pathway controls RhoA expression. In rat aortic and human internal mammary artery smooth muscle cells (SMC), cGK stimulation by NO donor (sodium nitroprusside (SNP), 10 μ*M*) or by specific cGK activator (8-pCPT-cGMP, 50 μ*M*) increased both RhoA mRNA level (2.7-fold) and RhoA protein expression (3.1-fold). These effects were inhibited by pretreatment with the cGK inhibitor Rp-8-Br-PET-cGMPS (100 n*M*). Furthermore, analysis of RhoA activity by pull-down assay showed that the cGK-induced rise in RhoA expression was associated with a 4.3-fold increase in the amount of active GTP-bound RhoA. Activation of NO/cGK pathway had no effect on RhoA mRNA stability but increased the RhoA protein half-life (5.9 ± 0.2 h (n = 4) versus 9.1 ± 0.3 h (n = 4) in control). Transfection experiments demonstrated that the half-life of the $RhoA^{ala188}$ mutant that could not be phosphorylated by cGK was not prolonged by the activation of the NO/cGK pathway. In conclusion, these results show that cGK increases RhoA expression by stimulation of *rhoA* gene transcription and stabilizes RhoA protein through direct phosphorylation of RhoA on Ser188. Arterial disease such as atherosclerosis and hypertension are known to be associated with alteration of NO/cGK and RhoA signaling pathways. Our work demonstrates a new link between RhoA and NO/cGK pathways that can be involved in these pathogenic processes.

Sphingosine Kinase Modulates Microvascular Tone via Activation of The RhoA/Rho Kinase Pathway

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There is growing evidence that RhoA and Rho kinase are important modulators of microvascular tone. Here we tested whether endogenous sphingosine-1-phosphate (S1P) stimulates this pathway in resistance arteries (RA) and whether this lipid mediator affects resting tone and myogenic responses (MR). RA were kept in organ culture and incubated for 19–22 h with plasmids coding for the S1P-generating enzyme sphingosine kinase (wild type Sphk: wtSphk) or its inactive mutant hSK-G82D. Dominant active RhoA (L63RhoA) or an inactive RhoA mutant (N19RhoA) or inactive Rho kinase (KD1A) were coexpressed with wtSphk to assess the involvement of the RhoA/Rho kinase pathway in mediating Sphk effects. GFP (green fluorescent protein)-transfected RA, that showed vascular responses virtually identical to untransfected RA, served as controls. Resting tone (rT) was significantly enhanced in RA expressing wtSphk (by 23 ± 3% of max. diameter vs. 10 ± 1% in RA_{GFP}, $p < 0.01$) and L63RhoA (by 22 ± 2%) but almost abolished in hSK-G82D-transfected RA (by 2%). Coexpression of N19RhoA or KD1A with wtSphk abolished development of rT. MR (initiated by pressure steps from 45 to 110 mm Hg) were enhanced after transfection of wtSphk and L63RhoA (154 ± 14% and 92 ± 12% of initial distension vs. 61 ± 8% in RA_{GFP}, $p < 0.01$) but almost completely inhibited after coexpression of N19RhoA or KD1A or transfection with hSK-G82D. The ability of endogenous Sphk to activate the RhoA/Rho kinase pathway via its product S1P implicates a possible important role for the this enzyme as a modulator of microvascular resting tone and myogenic reactivity.

Rho Kinase Is Importantly Involved in Renal Vasoconstriction

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Various hormones, which activate distinct signaling pathways, regulate glomerular filtration via controling glomerular hemodynamics through preglomerular vessels and efferent arterioles. Though it has been established that the Rho/Rho kinase (ROK) pathway can induce Ca^{2+} sensitization via inactivation of myosin light chain phosphatase, it is unknown whether ROK contributes to renal vessel tone. In the present study we therefore measured the impact of ROK inhibition on the microcirculation of the split hydronephrotic rat kidney in vivo. Local application of the ROK inhibitor Y-27632 (0.1–100 μ*M*) induced marked dilation of renal vessels by up to 40%, and increased glomerular blood flow (GBF) by 98 ± 13% (n = 19). The structurally different ROK inhibitor HA-1077 exerted similar effects (n = 5). To study the importance of ROK for renal vasoconstrictors, the ET_B receptor agonist IRL 1,620 (10 n*M*), which is known to activate phospholipase C and RhoA, was applied before and after ROK inhibition with Y-27632 or HA-1077 (n = 6 and 5). ROK inhibition, but not protein kinase C inhibition with Ro 31-8220 (5 μ*M*, n = 5), blunted ET_B-induced vasoconstriction in preglomerular vessels and efferent arterioles. Furthermore, the reduction of GBF and of vascular diameters in response to guanylyl cyclase inhibition (30 μ*M* ODQ, n = 6) and to inhibition of adenylyl cyclase by adenosine A_1 receptor stimulation (10 μ*M* CPA, n = 7) were abolished by pretreatment with Y-27632. Though ROK inhibitors diminish stress fibers in cultured cells, confocal microscopy demonstrated that Y-27632 did not change F-actin content and distribution in renal vessels. Our results demonstrate that, in the kidney, ROK is importantly involved in mediating preglomerular as well as efferent vasoconstriction.

Endothelin-1-Induced Vasospasms of the Spiral Modiolar Artery Are Mediated by a Rho-Kinase-Induced Ca^{2+} Sensitization of the Contractile Apparatus and Reversed by CGRP

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Background: Vasospasms of the spiral modiolar artery (SMA) may cause an ischemic stroke of the inner ear that manifests itself by a sudden hearing loss. Previously we have shown that endothelin-1 (ET-1) induces vasospasms of the SMA. Here we tested the hypotheses that ET-1-induced vasospasms are a) reversible by ET_A receptor antagonists, b) mediated by a Ca^{2+} sensitization of the contractile apparatus via a Rho-kinase-induced inhibition of myosin light chain phosphatase (MLCP), and c) reversible by the second messenger cAMP and the vasodilator CGRP. **Methods:** The Ca^{2+} sensitivity of the contractile apparatus was evaluated by a correlation between the smooth muscle cell Ca^{2+} concentration and the vascular diameter that were measured by fluo4-microfluorometry and videomicroscopy, respectively. **Results:** ET-1-induced vasospasms were prevented but not reversed by the ET_A receptor antagonists BQ-123 and BMS-182874. The Ca^{2+} sensitivity of the contractile apparatus was increased by ET-1 and by inhibition of MLCP with calyculin A and decreased by calcitonin gene-related peptide (CGRP). ET-1-induced vasospasms and Ca^{2+} sensitization were prevented and reversed by the Rho-kinase antagonist Y-27632, by CGRP, and by the cAMP analog dbcAMP. **Conclusions:** ET-1 induces vasospasms of the SMA via ET_A receptor-mediated activation of Rho-kinase, inhibition of MLCP, and an increase in the Ca^{2+} sensitivity, which is reversed by CGRP and cAMP. The observation that vasospasms were reversed by Y-27632 but not by BQ-123 or BMS-182874 suggests that Rho-kinase, rather than the ET_A-receptor, is the most promising pharmacological target for the treatment of ET-1-induced vasospasms, ischemic strokes and sudden hearing loss.

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Sphingosine-1-Phosphate: A New Regulator in ROS Formation of Vascular Smooth Muscle Cells

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We have previously shown that depolarisation enhances the production of reactive oxygen species (ROS) in vascular cells. Here we studied whether increases in transmural pressure that are known to depolarise the vascular smooth muscle promote ROS formation and whether sphingosine-1-phosphate (S1P) that mediates contractile responses to increases in transmural pressure is also involved in formation of ROS. Using the DCF method we showed that an increase in transmural pressure (from 45 to 100 mm Hg) resulted in a significant increase in ROS formation of smooth muscle cell (SMC) layer of intact isolated resistance arteries (hamster, n = 4). Likewise, S1P treatment also enhanced the ROS formation of SMC in isolated vessels as well as in cultured SMC (n = 3, n = 6, resp). To further analyse a potential role of S1P in pressure-induced ROS formation, its generating enzyme, sphingosine kinase was overexpressed in isolated arterioles, either as wild type (Sphk1) or as a dominant-negative mutant (hSK^{G82D}). Overexpression of hSK^{G82D} significantly inhibited the pressure-induced ROS formation by SMC (n = 5), while the ROS formation induced by external S1P was not affected. The pressure-induced ROS production in Sphk1-overexpressing arteries could be inhibited by the NADPH-oxidase-blocking peptide gp91 ds tat. In conclusion, our data show that an increase of transmural pressure leads to an enhanced ROS production of SMC. Our data suggest that endogenous S1P generated by sphingosine kinase (Sphk) is involved in this process because genetic inhibition of Sphk reduces the pressure-induced ROS formation. Furthermore, S1P mimics the effect of pressure elevation. The inhibiting effect of the gp91ds peptide suggests the vascular NADPH oxidase to be the source of the ROS produced upon stimulation with S1P.

Poster Presentations

(ordered according to sessions)

Hydrogen Peroxide Induces E-Selectin in Human Endothelial Cells Independent of Nuclear Factor-κB Activation

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Background: Reactive oxygen species may be inflammatory signal mediators. We investigated if H_2O_2 induces expression of adhesion molecules (AM) and adhesion of monocytic cells in human umbilical vein endothelial cells (HUVEC). **Methods:** Detection of mRNA was by semi-quantitative RT-PCR; measurement of AM expression was by flow cytometry (FACS), activation of transcription factors AP-1 or NF-κB by electrophoretic mobility shift assay (EMSA). We used PD098059 (PD) and SB203580 (SB), specific inhibitors of the MAP kinase pathway, or MG132, an inhibitor of NF-κB. Adhesion assays were performed with 30 min co-incubation of HUVEC and monocytic U937 cells. **Results:** After stimulation with 200 μM H_2O_2, mRNA for ICAM-1 and E-selectin was increased, with a maximum at 6 h. FACS analysis demonstrated increased E-selectin and ICAM-1 protein, but no VCAM-1. H_2O_2 enhanced E-selectin-dependent adhesion of U937 cells to HUVEC, indicating upregulation of functional AMs. PD and SB reduced H_2O_2-induced E-selectin and ICAM-1 protein expression, and adhesion of U937 cells to HUVEC. H_2O_2 activated AP-1, but not NF-κB, while TNF-α (10 U/ml) induced both, and increased the expression of all three AMs tested. **Conclusions:** H_2O_2 induces MAP kinase-dependent AP-1 activation, which leads to expression of E-selectin, and increased adhesion of monocytes to endothelium. This mechanism may play a role in monocyte recruitment in conditions such as inflammation, re-perfusion, and atherosclerosis.

Albumin Infusion Modulates the Local Inflammatory Response in the Mesenteric Microcirculation following Abdominal Surgery

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Objective: The present study was designed to evaluate the effect of two different low-dose albumin regimens on the local inflammatory response following abdominal surgery. Albumin loss during surgery is a well described phenomenon. In previous experiments a loss

of plasma proteins, resp. albumin was observed during abdominal surgery. Intravital microscopy for five hours was used to evaluate the effect of low dose albumin on the mesenteric microcirculation. **Methods:** Urethan-anesthetized Sprague-Dawley rats underwent median laparatomy and placement of a doppler flow probe around the abdominal aorta. An ileal loop was prepared for eventration onto a microscopic stage using a plastic foil technique and the mesentery was immersed with Krebs-Henseleit buffer (5% CO_2 in N_2). During the experiment low dose albumin (1 mg/(kg BW·h)) was given continuously (CD) vs. a two-step dosage (DP) (2.8 mg/(kg BW·h) for 2 h, 0.28 mg/(kg BW·h) for 5 h) matching the loss profile of albumin as measured in previous experiments. Heart rate, MAP, aortic blood flow were registered on a beat-to-beat basis. ABG's were drawn hourly for analysis of metabolic(BE), respiratory (pO_2, pCO_2) and hct values. **Results:** Rolling leukocytes constantly remained on a low level in the DP group, whereas a significant rise in Rollers could be noted in the CD group after 120 min. Velocity, shear rate and stickers in the mesenteric microcirculation did not differ significantly between the two groups. However a significant rise in MAP could be seen in the CD group at the end of the experiment. **Conclusion:** A 'profiled dosage' of albumin significantly reduces the number of rollers in the mesenteric microcirculation following abdominal surgery. However the higher amount of rollers under continuous dosage does not necessarily lead to a significant rise in the number of stickers.

Expression of Cell Adhesion Molecules in Appendicitis: An in situ Model to Analyse Vascular and Microvascular Endothelial Cells

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Introduction: Cell adhesion molecules (CAM) regulate the recruitment and migration of leucocytes and play a crucial role in the pathomechanisms of inflammatory and immunological diseases. Their functional role has already been demonstrated in many in vitro systems and various animal models. We analyse the expression of intercellular cell adhesion molecule (ICAM-1), vascular cell adhesion molecule (VCAM-1) and E-selectin in normal appendices and specimens from different stages of appendicitis. **Methods:** Frozen sections of normal appendices from different stages of appendicitis (n = 71) corresponding to 6, 12, 24–48 and more than 72 h after appearance of clinical symptoms were analysed by histological and immunohistological methods. **Results:** Endothelial cells in normal appendices demonstrated only an expression of ICAM-1. Inflamed appendices demonstrated an early E-selectin expression (6 h) with a steady increase up to 24 h and a moderate reduction after 48 h, followed by the expression of VCAM-1. We found an inhomogenous expression pattern for E-selectin with strongest expression in veins, followed by arteries. The lowest expression could be demonstrated in capillaries. **Discussion:** The present study demonstrates the kinetics of CAM expression in endothelial cells at different stages of inflammation. Our results underline the different time course of CAM expression in situ compared with in vitro data and demonstrate that in vitro data are not entirely representative of the in vivo situation. Furthermore, the divergent expression pattern within the different segments of the cardio-vascular system indicates the phenotypic and functional differences of microvascular endothelial cells. In conclusion we present an interesting in situ model, which allows the simultaneous observation of vascular and microvascular endothelial cells under simultaneous conditions and facilitates the investigation of the role of these cells in the regulation of inflammatory reactions.

Activated Human Dermal Microvascular Endothelial Cells (HDMEC) Can Be Separated into Subpopulations with Distinct Expression Patterns Using the Thy-1 (CD90) as Selection Marker

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Microvascular EC represent a heterogeneous population differing along blood and lymph vessels, in various organs and fulfilling various functions. Reliable cell markers are the major prerequisite for the detection and analysis of those subpopulations. We have demonstrated that human Thy-1 is an activation marker on a subpopulation of HDMEC. These subpopulations can be separated using antibody coupled microbeads and analysed towards cell biological functions and expression patterns. Microarray analysis was performed to identify differentially expressed genes in Thy-1 positive vs. Thy-1 negative cells. Two RNA pools of Thy-1(+) and Thy-1(–) activated HDMEC each consisting of 5 independent separations were used for 4 hybridisations of Affymetrix U95 chips. The hybridisation intensity data were compared from all pools generated among each other (4 comparative analyses). About 100 genes showed expression differences of >2 fold after each comparison of Thy-1(+) vs. Thy-1(–) cells. The data were strongly reproducible in Realtime-RT-PCR analysis for highly differently expressed genes (32 fold increase) as well as for weakly increased genes (~2 fold increase) proving the reliability of the data from the microarray experiment. Among them we found cytokines (IL-7), growth factors (bFGF, glycodelin) and their receptors (VEGFR-2), adhesion molecules (CD209, integrin α9), matrix degrading enzymes (MMP-9, uPA) and gene products involved in inflammation (iNOS, MMR) and immune response (MHCII-DP-βchain). Moreover, these mRNA could be detected in situ in endothelial cells in the human skin. Using Thy-1 as an activation marker of certain HDMEC we are able to identify and characterise new activation associated gene products.

The Thy-1 Molecule on Activated Human Dermal Microvascular Endothelial Cells Specifically Interacts with CD11b (αM-Integrin) on Leukocytes – Impacts on Inflammatory Processes

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Our previous work demonstrated that human Thy-1 is a newly identified activation-associated cell adhesion molecule on human dermal microvascular endothelial cells (HDMEC). Thy-1 is involved in the adhesion of monocytes and polymorphonuclear cells (PNC) to activated HDMEC. In this study we identified the corresponding ligand of the Thy-1 molecule on these cells. First, we generated a monoclonal antibody (mAb AW2) against PNC and monocytes blocking the binding of Thy-1 protein to its ligand. This antibody shows the same binding pattern as an anti-CD11b antibody. Blocking experiments revealed that the mAb AW2 inhibits the binding of an anti-CD11b antibody to monocytes. Second, purified and labeled Thy-1 protein binding to its ligand on monocytes and PNC has a similar binding profile as the anti-CD11b antibody and mAb AW2 in flow cytometry. Additionally, purified Thy-1 protein was able to reduce binding of anti-CD11b antibody as well as mAb AW2 suggesting that Thy-1 binds to CD11b (αm-chain of integrins) on PNC and monocytes. Cell adhesion studies showed that mAb AW2 as well as anti-CD11b antibodies were able to block adhesion of PNC and monocytes to Thy-1-transfected COS cells emphasizing an essential role of Thy-1/CD11b interaction for cell adhesion. The CD11b is a well characterized cell adhesion molecule on monocytes and PNC. Until now it is thought that ICAM-1 is the main cellular ligand of CD11b. Our data first time provide evidence that human Thy-1 on activated HDMEC is a second cellular ligand of CD11b mediating the binding of monocytes and PNC to activated endothelium, possibly regulating invasion of leukocytes into inflamed tissues.

Syk-Kinase Controls Migration of Human Neutrophils via the Binding Cycle of the β_2 Integrins (CD11/CD18)

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During recruitment of human polymorphonuclear neutrophils (PMN) to sites of inflammation, leukocyte adhesion molecules of the β_2 integrin family (CD11/CD18) mediate firm adhesion of these cells to the endothelial cell monolayer lining the vessel wall. A fine control of the binding of the β_2 integrins to their ligands is required to allow shape change and spreading as well as migration of PMN on the endothelial cell monolayer to the intercellular junction between neighbouring endothelial cells where PMN eventually transmigrate into the extravascular space. To elucidate the molecular mechanisms which allow ordered β_2 integrin-mediated adhesion and de-adhesion of PMN, intracellular protein tyrosine signalling was studied subsequent to β_2 integrin-mediated ligand binding. By means of immunoprecipitation and Western blotting technique, a 72-kDa protein which was tyrosine phosphorylated upon adhesion was identified as Syk-kinase. Moreover, Syk-kinase was found to be directly associated with CD18, the β-subunit of β_2 integrins. Inhibition of Syk-kinase by piceatannol enhanced adhesion and spreading of PMN but diminished fMLP-induced chemotactic migration. Inhibition of tyrosine phosphatases by vanadate inhibited adhesion and it also diminished fMLP-induced chemotactic migration. The analysis of the molecular mechanism which underlies the enhancement of PMN adhesiveness revealed that inhibition of Syk-kinase induced the clustering of the β_2 integrins on the cell surface which is known to result in increased integrin avidity. In contrast, inhibition of Syk-kinase had no effect on CD18 expression or expression of activation-specific neoepitopes on β_2 integrins. Altogether, the present study suggests that Syk-kinase may control alternation of strong β_2 integrin-mediated ligand binding with integrin detachment which is necessary to allow ordered migration of PMN to sites of inflammation by controlling the avidity of the β_2 integrins via integrin clustering.

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A Novel Method for the Quantitative Microscopic Analysis of Leukocyte Migration in vivo

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Transendothelial migration plays a key role in the multistep recruitment of leukocytes to sites of inflammation. We established a microscopic method allowing the in vivo analysis of all steps of leukocyte migration. Leukocyte rolling, adhesion, and transmigration were investigated in the cremaster muscle of C57BL/6 mice under control as well as under inflammatory conditions after topical application of either PAF (platelet activating factor, 100 n*M*) or KC (murine chemokine acting on CXCR2, 6.25 n*M*). Conventional intravital fluorescence microscopy was combined with near-infrared reflective transillumination. Leukocyte-endothelial cell interactions (rolling and adhesion) were visualized conventionally after intravital labelling of leukocytes with rhodamine 6G. Leukocyte transmigration was observed by transillumination using monochromatic near-infrared light of a single wavelength of 683 nm. This newly developed transillumination method provides deep tissue penetration and pseudo-three-dimensional plasticity of cellular structures in combination with less absorption of hemo- and myoglobin and low energy transfer. Stimulation with either PAF or KC decreased the number of rolling leukocytes (n/30 s) after 30 min from 17.4 ± 2.6 (control) to 4.8 ± 0.5 (PAF) and 9.8 ± 2.8 (KC). In contrast, the number of adherent leukocytes ($n/10^4\ \mu m^2$) significantly ($p < 0.05$) was increased from 3.6 ± 0.9 to 18.6 ± 1.3 and 9.6 ± 1.8, respectively. After 60 min, this was followed by a significant increase in the number of emigrated leukocytes ($n/10^4\ \mu m^2$) from 5.0 ± 1.6 to 22.5 ± 2.3 and 13 ± 2.2, respectively. In summary, by the combination of conventional fluorescence microscopy with near-infrared light reflective transillumination we present a microscopic approach to an in vivo study of dynamic interactions during the complete leukocyte emigration process in mice.

Differences in CCR5 and CCR3 Expression on CD4+ and CD8+ T-Cells between Localized and Generalized Wegener's Granulomatosis

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Chemokine receptors mediate recruitment of T-cells at sites of inflammation. Little is known on the role of chemokines and their receptors in Wegener's granulomatosis (WG), an autoimmune disease of unknown etiology characterized by granulomatous and vasculitic lesions. We analyzed CCR5 and CCR3 cell surface expression on CD4+ and CD8+ T-cells by four-color flow cytometric analysis (FACS) in localized WG (n = 5), i.e. WG restricted to the respiratory tract, generalized WG (n = 16), and age- and sex-matched healthy controls (n = 13). The fractions of Th1-type CCR5+ and Th2-type CCR3+ cells within the CD4+CD45RO+ and CD8+CD45RO+ memory T-cell populations were significantly expanded in localized and generalized WG. The mean percentage of CCR5 expression on T-cells was highest in localized WG. T-cells lacking the co-stimulatory molecule CD28 preferentially displayed CRR5 expression. In contrast, T-cells displayed similarly up-regulated levels of CCR5- and CCR3 expression on T-cells in generalized WG. An immunohistochemical study demonstrated higher numbers of CCR5+ cells in granulomatous lesions of localized WG as compared to generalized WG. Thus, up-regulated CCR5 and CCR3 expression on memory T cells indicates activation and homing capability of CD4+ and CD8+ memory T cells in WG. Earlier studies showed a predominantly Th1-type cytokine pattern in granulomatous lesions of the respiratory tract in localized WG and a shift towards a Th2-type cytokine pattern in generalized WG. Higher levels of CCR5 cell surface expression may favor a stronger CCR5-mediated recruitment of Th1-type memory effector T-cells into granulomatous lesions of the respiratory tract in localized WG as compared to generalized WG.

Differences in Spheroid-Like Granuloma Formation between Wegener's Granulomatosis (WG) and Systemic Lupus erythematodes (SLE)

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Necrotizing granulomas, accompanied by geographic necrosis and vasculitis are the pathological hallmarks of WG. To examine mechanisms and functions of granulomas and their formation we designed an in vitro granuloma model based on spheroid models in tumor biology. Isolated PBMC and PMN from healthy controls (HC; n = 4), generalized WG (n = 6) and SLE (n = 4) were added onto a HUVEC monolayer in a transwell over agarose-coated wells, stimulated (SEE + PR3-ANCA IgG) and cultured for four days. Phenotype (CD3, CD20, CD26, CD28, CD54, CD163) and functional features (MIP-1β, osteopontin, TNFα) of the spheroid-like granuloma cultures were determined by immunohistochemistry and ELISA. The mean number of in vitro granulomas was 12/12 for WG, but only 1/11 for SLE ($p < 0.05$) and 5/12 for HC ($p < 0.05$) as assayed by microscopy. Leucocyte migration through the HUVEC monolayer took place in all cases and may have been directed by MIP-1β, which was not substantially different between WG, SLE and HC (8–10 ng/ml). Differentiated monocytes (CD163+) and CD26+ T cells were found in active WG. In contrast, in vitro granulomas from inactive disease displayed mainly CD3+ and CD28+ cells, but only few if any CD163+ or CD26+ cells. In addition, TNFα production by migrated leucocytes and endothelial cells was reduced in WG (0.7 ng/ml) when compared to SLE (1.7 ng/ml, $p < 0.05$) and HC (1.6 ng/ml). The model described herein suggests that peripheral leucocytes contain disease-specific cells or cellular subsets leading to the formation of an increased number of in vitro granulomas in WG.

Failure of Vitamin C – Alone or in Combination with the Iron Chelator Desferal – to Attenuate the Microcirculatory Dysfunction in Response to Hemorrhagic Shock and Resuscitation in Hamsters

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In previous studies on local muscle ischemia/reperfusion in the dorsal skinfold chamber model in hamsters, we demonstrated that post-ischemic leukocyte adhesion to postcapillary venules as well as break-down of endothelial barrier function and nutritional capillary perfusion were markedly attenuated by the combined treatment with vitamin C and desferal, while in turn vitamin C per se promoted leukocyte adhesion. In the present study, we have tried to transfer this protective effect to a hemorrhagic shock model in the same animal species. **Methods:** 28 hamsters were fitted with dorsal skinfold chambers and leukocyte adhesion in postcapillary venules and arterioles (Rhodamin 6G), functional capillary density and transendothelial leakage (FITC dextran) were assessed in striated muscle by intravital microscopy. Values were assessed at baseline and at 2, 24 and 72 h after a 2 h hemorrhagic shock period (40 mm Hg held constant by removal of 2.5–3.0 ml blood, which was anticoagulated with citrate, stored at 4°C, and re-infused via indwelling catheters for effective resuscitation). **Results:** Hamsters fed for 1 week prior to the experiments with a vitamin C rich diet (10 g/kg diet) showed increased post-resuscitation leukocyte adhesion to venules (72 h: 248 $\pm$ 89 vs. 143 $\pm$ 63 cells/mm^2 in controls, n = 7 each group; $p < 0.05$, Wilcoxon). Likewise, transendothelial leakage was significantly increased ($p < 0.05$). A similar aggravation of these parameters was seen in animals injected desferal (50 mg/kg body weight) immediately prior to resuscitation (not shown). In contrast to our previous observations in the local ischemia/reperfusion injury model, we found that the combined administration of vitamin C and desferal significantly aggravated – rather than attenuated – the micro-vascular dysfunction after generalized hemorrhagic shock (adherent leukocytes at 72 h: 334 $\pm$ 63 cells/mm^2, $p < 0.0025$ vs. controls, $p < 0.05$

vs. vitamin C or desferal monotherapy). Functional capillary density was not affected by any of the treatment modalities. **Conclusion:** Data from one model of (local) ischemia/reperfusion injury cannot necessarily be transferred to another model of (systemic) i/r injury, even using identical treatment protocols and the same animal species.

Acetylcholinesterase – a New Mediator of Leukocyte-Endothelium Interactions

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The extra-neuronal localisation of acetylcholinesterase (AChE) in leukocytes and vascular endothelium has supported the hypothesis of this protein have other functions besides the catalytic one. At level of the microcirculation, velnacrine maleate (an AChE inhibitor) increases the LPS-induced inflammatory response in Wistar rats, increasing the number of adherent leukocytes to endothelium of mesenteric post-capillary venules. The interaction between leukocytes and endothelium is regulated by cytokines such as IL-1β and TNFα, and the cAMP is one of the second messengers controlling the synthesis of these cytokines but also mediating its function in target cells. Cellular expression AChE is also modulated by cellular [cAMP] and the AChE/inhibitors interaction results in a decrease of cellular [cAMP]. The aim of this study was to verify if the velnacrine maleate interacting with AChE induces an increase of leukocyte-endothelium interactions by increasing cAMP-dependent cytokine release to blood circulation. **Methods:** Wistar rats (n = 30) were prepared in order to study the effect of velnacrine maleate on the leukocytes rolling and adhesion to endothelium in cremaster post-capillary venules using intravital microscopy and IL-1β and TNFα plasma concentrations were determined by ELISA, in presence and absence of trequinsin, an inhibitor of cAMP phosphodiesterase. **Results:** It was verified that velnacrine maleate significantly increased the number of rolling leukocytes in post-capillary venules ($p < 0.004$) and plasma [IL-1β], being this effect inhibited in the presence of trequinsin ($p < 0,01$). **Conclusion:** These results confirm the hypothesis that velnacrine maleate interacting with AChE induces an increase of leukocyte-endothelium interactions (rolling) by increasing cAMP-dependent cytokine (IL-1β) release to blood circulation.

Impact of Intraischemic Organ Temperature on Oxidative Stress during Hepatic Reperfusion

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The mechanisms of liver tissue protection from microvascular ischemia-reperfusion (I/R) injury by mild hypothermia are controversially discussed. This study was designed to investigate the influence of ischemic temperature on oxidative stress during hepatic reperfusion. In C57BL/6 mice, ischemia of the left liver lobe was induced for 90 min and ischemic temperature adjusted to 37 °C, 26 °C, 15 °C, and 4 °C by superfusion with cooled/warmed NaCl (n = 5 each group). After 5 and 30 min of reperfusion, hydroxyl radicals (spin trap DEPMPO) and ascorbyl radicals (direct detection) were quantified in plasma using electron spin resonance spectroscopy. Thiobarbituric acid-reactive materials (TBARMs) were detected in liver tissue as an indicator of lipid peroxidation. Intravital microscopy was performed to analyze leukocyte-endothelial cell interactions in postsinusoidal venules. In the sham-operated group, low levels of DEPMPO-OH adducts and ascorbyl radicals were detected (5.1 ± 0.2 μ*M* and 40.6 ± 1.9 μ*M*). Significantly increased levels of DEPMPO-OH and ascorbyl radicals were measured after ischemia at 37 °C and reperfusion for 5 min (12.6 ± 0.9 and 94.2 ± 22.1 μ*M*) and 30 min (10.6 ± 0.9 and 89.0 ± 9.7 μ*M*). After ischemia at 4 °C, 15 °C, and 26 °C, however, plasma concentrations of free radicals did not differ from those after ischemia at 37 °C. TBARMs were not detected in the sham-group as well as in the groups after ischemia at 4 °C and 15 °C. In contrast, TBARM tissue concentration was slightly increased after 26 °C and markedly elevated after ischemia at 37 °C (0.5 ± 0.1 nmol/g, $p < 0.05$). Leukocyte adherence was enhanced in the group at 37 °C (194 ± 39/mm^2), whereas it was attenuated after hypothermia at 26 °C (13 ± 9/mm^2, $p < 0.05$). In addition, procoagulant plasma activity of thrombin, which triggers the CD62P-dependent leukocyte recruitment, was reduced in the hypothermic groups. Our data demonstrate that mild hypothermia protects the liver tissue from I/R-induced oxidative stress. This protective effect is associated with an attenuation of leukocyte adherence in hepatic microvessels, but not of plasma levels of free oxygen radicals.

Differential Role of NO, CO and Biliverdin in Mimicking Cryoprotection of Inflamed Tissue

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Introduction: Local cooling of inflamed tissue exerts well-known protective effects. In previous studies we could show that hemeoxygenase-1 and NO synthase are involved in this process. The aim of the present study was to evaluate to what extent the individual mediators of both pathways, i.e. NO, CO and biliverdin, contribute to the maintenance of microcirculation and the protection from cellular injury in TNF-α-exposed tissue. **Materials and Methods:** Skinfold chambers of hairless mice were locally exposed to TNF-α (controls). Volumetric blood flow (VBF), capillary density (FCD), leukocyte adherence and apoptosis were analyzed in vivo before and up to 180 min after TNF-α exposure. In additional animals the chamber was cooled for 30 min immediately upon TNF-α exposure. Animals of further groups were pretreated with either L-arginine, hemin or Trolox, followed by TNF-α exposure, however without cooling. **Results:** In TNF-α-exposed controls, VBF and FCD decreased while leukocyte adherence and apoptotic cell death increased. Cooling prevented these TNF-α-induced injuries. Treatment with L-arginine inhibited perfusion failure and apoptotic cell

death while leukocyte adherence was similar to that in controls. HO-1 induction by hemin protected perfusion, abrogated leukocyte adherence but only slightly reduced apoptotic cell death. Trolox could not prevent TNF-α-induced microcirculatory dysfunction while it reduced apoptotic cell death and completely supressed leukocyte adherence. **Conclusion:** NO and CO contribute to the cooling-mediated protection of TNF-α-injured tissue against microcirculatory failure, inflammation and apoptotic cell death as supplementation of these mediators was able to mimic tissue cryoprotection. The antiadhesive effect of cooling seems to be mainly mediated by the HO-1-derived antioxidative metabolite biliverdin.

Activated Platelets Synthesize IL-1β Directly from Megakaryocyte-Derived mRNAs in a p38 MAP-Kinase-Dependent Fashion

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Platelet adhesion to damaged endothelium or the subendothelium is one factor by which platelets promote the development of atherosclerosis. It is generally believed that these pathophysiologic functions depend largely on the release of preformed mediators. Here, we demonstrate that platelets may also contribute to prolonged inflammatory events by synthesizing new proteins. Using gene array analysis, we found that platelets contained numerous megakaryocyte-derived mRNAs. By ^{35}S-methionine uptake, we demonstrated that platelets translate mRNAs into protein in an activation-dependent fashion. With confocal microscopy we have shown that platelets contain a complete and physiologically functional translational apparatus. We have further shown that this translational machinery becomes functionally active with the cytoskeletal rearrangement upon aggregation. One of the mRNAs that is translated into protein in activated platelets is interleukin-1β (IL-1β), which we found in the polysome fraction of activated platelets. Following stimulation with PAF (10 n*M*) or thrombin (0.1 U/ml) the IL-1β precursor mRNA was translated into protein (unstimulated control (co): 23 ± 11 pg/ml; PAF (10^{-7} *M*, 8 h): 2,374 ± 259 pg/ml; $p \leq 0.05$ vs. co). The IL-1β precursor protein was further processed and secreted as active, mature IL-1β within the microvesicle fraction (co: 15 ± 9 pg/ml; PAF: 446 ± 223 pg/ml; $p \leq 0.05$ vs. co). We also found that the p38 MAP-kinase was phosphorylated within minutes and remained phosphorylated up to several hours. Pretreatment of platelets with the p38 MAP-kinase inhibitor SB203580 prevented the synthesis of IL-1β. In conclusion, this study provides data, which define a new role for platelets in inflammation and indicate that regulated protein synthesis may be a mechanism by which platelets can influence inflammatory outcomes.

Antiinflammatory and Antiproliferative Properties of Antithrombin

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Background: The serpin antithrombin (AT) is reported to have not only anticoagulatory but also antiinflammatory and antiangiogenic properties. The molecular mechanism underlying these effects is still unclear. In the present study, we investigated the influence of AT on NFκB and MAPK signal transduction pathways in endothelial cells (EC) and monocytes (MO), both well-known proinflammatory signaling pathways. Furthermore we studied the effect of AT on the proliferation of cultured smooth muscle cells. **Methods and Results:** EC and MO were incubated with TNF-α and LPS, respectively, in presence and absence of increasing concentrations of AT. AT inhibited the activation of the transcription factor NFκB in a dose-dependent manner by preventing phosphorylation and degradation of the inhibitory protein IκBα. Moreover, AT prevented the activation of the p54 subunit of SAPK. In parallel, the production of tissue factor, IL-6 and TNF-α, known to be under control of NFκB, were markedly reduced by AT (20% of control). The β-isoform of AT was more effective in preventing this proinflammatory activation than the α-isoform, whereas AT without heparin-binding site had no effect. AT showed a significant antiproliferative effect on cultured VSMC of about 25–35% inhibition. **Conclusions:** AT has shown to prevent NFκB- and MAPK-transactivation in EC and MO, suggesting a molecular mechanism of its direct antiinflammatory effect. Considering its antiproliferative effects on VSMC, AT might serve as a promising therapeutic agent for vasoproliferative disorders.

Comparative Analysis of Platelet Isolation Techniques for the in vivo Study of the Microcirculation

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Objective: In vitro and in vivo studies using isolated platelets require that the cells used for testing are not activated by the isolation procedure. This ensures that the effects measured by the test are the result of the environment or the applied stimulus, but not an artefact due to activation by cell isolation. **Methods:** Herein, we analysed two different platelet isolation procedures (i.e. Sepharose column versus density gradient centrifugation) with special emphasis on cell activation, including flow-cytometric analysis of P-selectin expression, functional quantification of mechanical platelet retention, light microscopic assessment of platelet aggregation, and fluorescence microscopic determination of in vivo rat liver platelet-endothelium cell interaction. **Results:** Under resting conditions Sepharose column-isolated platelets showed a negligible fraction of only 2.7 ± 3.3% cells with P-selectin expression, and an appropriate response (33-fold increase) upon activation with thrombin receptor activating

peptide (TRAP). In contrast, density gradient centrifugation resulted in P-selectin expression under resting conditions of already ~ 50% with an only 1.6-fold increase upon further TRAP stimulation. In addition, density gradient- but not Sepharose column-isolated platelets showed increased mechanical retention and aggregation in vitro, as well as pronounced adhesion to hepatic venular endothelium in vivo. Interestingly, density gradient-isolated platelets additionally induced in vivo an increase of colocalisation of platelets with adherent leukocytes, indicating a general microvascular inflammatory response. **Conclusion:** Density gradient centrifugation- but not Sepharose column-isolated platelets are activated already under resting conditions and induce in vivo a platelet-leukocyte-endothelial cell-associated inflammatory response. Thus, we propose that the method of platelet isolation using the Sepharose column is superior to the density gradient centrifugation technique, and might therefore be preferred for in vitro and in vivo assays to study platelet function.

The Lymphatic System in the Dorsal Skinfold Chamber of the Syrian Golden Hamster in vivo

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The lymphatic network contributes to maintain tissue homeostasis and immunological function by transporting fluid, plasma protein, and cells from peripheral tissue via lymph nodes into the blood vascular system. In contrast to the blood circulatory system little is known about the lymphatic system. In particular suitable animal models are lacking. Therefore, the dorsal skinfold chamber model was investigated regarding the existence of a lymphatic system. To analyze the lymphatic network Syrian golden hamsters (n = 12) fitted with titanium chambers were used. FITC-dextran of different concentrations (5 or 25%) and different molecular weight (4,000, 40,000 or 150,000 Da) was used to contrast lymphatic vessels and measure lymphatic velocity. Intravital fluorescence microscopy enabled the quantification of diameter, velocity and branching order. Histology and electron microscopy supported the in vivo findings. Immediately after intradermal injection of FITC-dextran lymphatics were visible exhibiting valves. The diameters of lymphatic vessel (n = 189) ranged from 133 ± 5.4 µm (branching order 1) to 26 ± 4.0 µm (branching order 5). Using different molecular weights of FITC-dextran no significant differences regarding the velocity were measured (4,000: 327 ± 157 µm/s; 40,000: 391 ± 126 µm/s; 150,000: 378 ± 175 µm/s). Blood and lymphatic vessels could not clearly be differentiated by H&E stainings. However, endothelial cells of vessels with an irregularily shaped lumen containing no erythocytes in cross sections showed a weaker signal for CD31 staining as compared to endothelial cells of vessels containing erythrocytes. Electron microscopy after intradermal injection of Berlin blue revealed dye-containing vessels with interendothelial openings typical for lymphatics. In conclusion, this study could demonstrate a lymphatic network in the dorsal skinfold chamber model of the Syrian golden hamster.

A Newly Developed Technique for Selective Middle Cerebral Artery Infusion in Rats

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Selective catheterization of and infusion of drugs into individual cerebral arteries is often employed in patients suffering from vasospasm due to subarachnoid hemorrhage or embolic stroke. In the present study a model of selective middle cerebral artery (MCA) infusion in conjunction with MCA occlusion is described. The tip of a 6-0 nylon thread was heat-blunted and coated with poly-*L*-lysin. The thread was introduced into a microcatheter with the tip exceeding the catheter by approximately 1 mm. The catheter was introduced into the right common carotid artery of rats which were anaesthetized with isoflurane (2.0–2.5%) and equipped with a laser Doppler flowprobe (LDF) over the right parietal cortex. The catheter was advanced until strong resistance was felt and the LDF signal immediately dropped. The volume of ischemic damage was measured after 8–24 h of MCA occlusion with the catheter or with a silicone-coated nylon suture (4-0). In some animals, the MCA was perfused with blood (hemodiluted to give a hematocrit of 20%) or with a perfluorocarbon emulsion. In these animals alterations of LDF signal or tissue oxygenation were monitored, respectively. Ischemic brain damage due to MCA occlusion induced by the catheter amounted to 346 ± 60 mm^2 after 8 h and 447 ± 71 mm^2 after 24 h of catheter occlusion as opposed to 348 ± 37 mm^2 and 413 ± 38 mm^2 after thread occlusion. Increasing the infusion rates in a stepwise manner (100–400 µl/min) resulted in stepwise increases of the LDF signal and of the $ptiO_2$, respectively. Infusion of Evans blue through the MCA catheter clearly stained the main trunk as well as the branching vessels and the entire MCA territory. In the present study an approach to perform selective perfusion of the MCA territory of the rat is described. This approach may be worthwhile in future studies on the pathophysiology of ischemic or traumatic brain injury.

Angiogenesis Induction by Embryonic EPCs in Chronic Hindlimb Ischemia of Rabbits

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Induction of therapeutic arteriogenesis and angiogenesis has been reported by application of VEGF or bFGF protein or cDNA. More recent reports indicate that circulating adult endothelial progenitor cells (EPCs) are capable of contributing to capillary growth and improved blood flow in the ischemic tissue. Adult EPCs expanded ex vivo and reintroduced into the recipient organism appear as a promising therapeutic tool for otherwise intractable chronic vascular disease. Embryonic EPCs have been studied as an alternative approach, overcoming shortage of cell supply while displaying immunoprivilege due to lack of MHC I expression. Therefore, we used embryonic mouse EPCs to study induction of angiogenesis and arteriogenesis in

a rabbit hindlimb ischemia model. **Methods:** In rabbits (n = 5 per group), the external femoral artery was excised at day 0. At day 7, either saline solution, EPCs (5×10^6), or bFGF (50 μg) with or without VEGF (20 μg) were infused into the ischemic tissue retrogradely through the anterior tibial vein. At day 35, analysis of passage time of contrast agent from internal iliac artery to anterior tibial artery was performed in vivo. Angiographic analysis of collateral growth as well as histological assessment of capillary/muscle fiber ratio were conducted. **Results:** Compared to saline-perfused controls, EPC treated hindlimbs displayed an increase in capillary/muscle fiber ratio at day 35 (1.41 ± 0.09 vs. 0.87 ± 0.11). Moreover, an increased number of collaterals was detected (203 ± 21 vs. 110 ± 13% of number counted at day 7), and blood flow velocity increased from 100% (day 7) to 151 ± 5% in the EPC-treated group, whereas control levels increased to 110 ± 13%. bFGF and VEGF coapplication resulted in similar increases in capillary density, collateral growth and blood flow velocity. We conclude that mouse embryonic EPCs are capable of inducing arteriogenesis and angiogenesis in a rabbit model of chronic ischemia, similar to the effect observed with bFGF + VEGF. Whether embryonic EPCs mainly release growth factors and thereby induce angiogenesis and arteriogenesis, or utilize another angiogenic signaling pathway, remains to be determined.

Heterogeneity of Angiogenic Endothelial Cells in vitro

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Angiogenesis is a multi-step process involving migration, proliferation and a specific spatial arrangement of endothelial cells. Numerous in vitro test systems of angiogenesis have been proposed all focussing on cultured endothelial cells. Our current working hypothesis says that not all endothelial cells possess the same properties and that specific 'specialist cells' exist among the microvascular endothelial cell population which play a crucial role in fundamental steps of the angiogenic cascade. The aim of the study was to collect data on heterogeneity in endothelial morphology, particularly intercellular junctions, and endothelial function. Based on a realistic in-vitro model of angiogenesis derived from microvascular endothelial cells from the bovine corpus luteum, the angiogenic cascade was studied light, scanning and transmission electron microscopically and TUNEL-test for apoptosis was performed. Different morphological avatars of angiogenic endothelial cells – the structural equivalent of diverse endothelial capabilities – were identified. In the proliferative and migratory steps of the angiogenic cascade, endothelia developed invadopodial structures and form cytoplasmatic projections for cellular inter-activity. Specific cell contact structures were built-up or degenerated, respectively, in the later angiogenic phases when two- and three-dimensional capillary-like structures were formed and the non-sprouting, i.e., intussusceptional restructuring of these endothelial tubes occurred. Apoptosis was detected in specific phases of the angiogenic cascade only. Crucial steps of angiogenesis such as three-dimensional arrangement, lumen formation or intussusceptional remodelling initiate from specific cell groups only, thus emphasising the concept of structural and functional heterogeneity of microvascular endothelia.

Transfection of Human Endothelial Cells at Different Stages of Angiogenesis

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Introduction and Aim: Angiogenesis, the formation of new blood vessels by endothelial cells, is downregulated in adult mammals and vasculature is quiescent with the exception of angiogenesis in the female reproductive cycle and in numerous pathological conditions such as tumor growth. Targeting endothelial cells by gene transfer to inhibit angiogenesis, i.e., in tumor growth, raises the question for side effects on physiologically quiescent endothelium. The aim of this study was to determine transfection efficiencies of human endothelial cells at different stages of angiogenesis. **Material and Methods:** Angiogenic endothelial cells from the human neonatal foreskin at different stages of angiogenesis (initial endothelial proliferation; terminal formation of capillary-like structures) were transfected. Plasmid vectors with constitutive (CMV promoter) and endothelial specific promoters (Ets-1 promoter of two different lengths, E-selectin promoter), encoding for luciferase, were complexed with Superfect®, a cationic transfection reagent. Luciferase activity was determined by measuring luminescence of the cell lysate. Samples were standardized by their protein content. **Results:** Gene transfer was successful to endothelial cells at the initial stage of proliferation. Reporter gene expression after formation of capillary-like tubes was marginally higher than control expression, however clearly lower than expression of cells at stage of proliferation. Highest transfection efficiencies were found by using the CMV promoter and the Ets-1 promoter of 1.85 kb. **Conclusions:** The results indicate that in view of a gene therapy only highly proliferative endothelium can be transfected efficiently, whereas quiescent endothelium is resistant to gene transfer.

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Angiogenesis in vitro – Quantification of the Angiogenic Cascade

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The effect of pro- and anti-angiogenic factors is investigated in numerous in vitro models of endothelial cells. Quantification of the different steps of the angiogenic cascade is a prerequisite for comparison and objectivation of statements on the effect of applied factors. In vitro models for quantification of angiogenesis all focus on cultured endothelial cells. In most models some but not all steps of the angiogenic cascade are shown, however, the formation of capillary-like structures with a lumen is presented in a few studies only. The aim of this study was to establish a method to quantify angiogenesis in an in vitro model which can be used in routine investigations of

different laboratories and is cost- and time-efficient. Cells were isolated from the bovine corpus luteum and identified by different endothelial markers. In our model, endothelial cells in vitro undergo all stages of the angiogenic cascade, i.e., proliferation, migration, sprouting and linear side by side arrangement of endothelial cells followed by organisation into a three-dimensional capillary-like network with an internal lumen. The development of capillary-like structures in vitro takes place in a fixed sequence: first the formation of a network of endothelial cells arranged in lines can be observed. Then the number of the strings of this network decreases while their diameter increases and finally a lumen appears. For quantification angiogenesis in vitro was classified into 9 strictly defined stages. Morphometric quantification was carried out in cell cultures beginning at angiogenic stage 7, i.e., at the first appearance of capillary-like structures. Measurements were done in precisely defined segments of culture wells and documented by digital photography. Morphometry included measuring of the area capillary-like structures occupied, their length and the number of branching points. Evaluation of experiments was done repeatedly by different investigators. Results indicate that this method is reproducible and useful for professional routine investigation.

A Role of Human Neutrophils in Inflammation-Mediated Angiogenesis

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Human polymorphonuclear neutrophils (PMN) play an important role in host defense and inflammation. In the present study, we investigated whether PMN may also contribute to the induction of inflammation-mediated angiogenesis. Using an in vitro angiogenesis assay, we found that N-formyl-Met-Leu-Phe (fMLP)-activated human PMN released a pro-angiogenic entity within 1 h after stimulation which induced sprouting of capillary-like structures. The effect was comparable in size to the pro-angiogenic activity of 100 ng/ml of vascular endothelial growth factor (VEGF) or basic fibroblast growth factor (bFGF). By means of ELISA technique, we found that activated PMN released the pro-angiogenic factors VEGF and interleukin 8 (IL-8): Within 1 h after the onset of stimulation, the supernatant of the fMLP-activated PMN (5×10^6/ml) contained about 80 pg/ml of IL-8 and about 40 pg/ml of VEGF. Whereas the release if IL-8 was almost completely blocked in the presence of actinomycin D or cycloheximide, the inhibition of mRNA or protein synthesis had no effect on the release of VEGF. This suggests that the pro-angiogenic potential of PMN consists of two components: the de novo synthesis of IL-8 as well as the release of preformed VEGF. Accordingly, the pro-angiogenic effect of the supernatant obtained from activated PMN was markedly inhibited in the presence of neutralising antibodies against VEGF or IL-8. Altogether, the present study suggests that activated PMN promote the sprouting of capillary-like structures. Thus, human PMN may be involved not only in host defense but also in the process of repair by promoting angiogenesis via the release of the pro-angiogenic factors VEGF and IL-8.

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Mitogen-Induced Up-Regulation of the Intermediate Ca^{2+}-Activated K^+ Channel (IK1) in Human Endothelium

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Ca^{2+}-activated K^+ channels (K_{Ca}) are important regulators of endothelial function and have also been proposed to promote cell proliferation by enhancing membrane potential driven Ca^{2+}-influx. Here we tested whether the IK1 is involved in endothelial cell proliferation in response to the angiogenic factors bFGF and VEGF. IK1 function and expression were determined by use of the patch-clamp technique and quantitative real time RT-PCR in human umbilical vein endothelial cells. A 48-hour exposure to bFGF (50 ng/ml) or VEGF (50 ng/ml) resulted in a significant increase in ChTX and clotrimazole-sensitive IK1-currents (bFGF: 1.92 ± 0.43 pA/pF, $p < 0.01$; VEGF: 1.79 ± 0.67 pA/pF, $p < 0.05$ vs. control: 0.36 ± 0.09 pA/pF), which was paralleled by a sixfold and fourfold increase in IK1 expression, respectively. Inhibition of PKC by staurosporine (10 nM) and of MAPKK by PD98059 (25 µM) prevented bFGF and VEGF-induced up-regulation of IK1. Moreover, bFGF- and VEGF-induced cell proliferation was almost completely abolished in the presence of ChTX (100 nM) or clotrimazole (1 µM). In conclusion, the angiogenic factors bFGF and VEGF stimulate expression and function of IK1 in endothelial cells. This up-regulation of IK1 seems to promote mitogen-induced cell proliferation and might therefore represent a new therapeutic target to prevent angiogenesis.

Rac and PAK-1 Promotes Tissue Factor Upregulation by Thrombin in Pulmonary Aortic Smooth Muscle Cells

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Pulmonary hypertension is characterized by hypertrophy and enhanced proliferation of vascular cells as well as a prothrombotic state leading to pulmonary remodeling. The signaling pathways promoting the increased thrombogenicity of pulmonary vessels are not understood. The GTPase Rac is involved in multiple signaling pathways leading to cell migration and proliferation. However, a role of Rac in promoting the prothrombotic state in pulmonary remodeling is not clear. We therefore investigated whether Rac and Rac-regulated pathways play a role in the regulation of tissue factor (TF), the main activator of the extrinsic coagulation cascade, by thrombin in human pulmonary aortic smooth muscle cells (PASMC). Thrombin-induced TF mRNA and protein expression as well as procoagulant activity were abrogated in PASMC transfected with expression vectors coding for the dominant-negative RacT17N but was enhanced in the presence of constitutively active RacG12V. Thrombin also stimulated the phosphorylation of the Rac effector p21-activated kinase-1

(PAK1), as well as of p38 MAP kinase (p38MAPK), ERK1/2 and protein kinase B (PKB). Expression of RacG12V enhanced phosphorylation of PAK1, p38 MAPK and PKB, but not of ERK1/2. Thrombin-induced phosphorylation of PAK1, p38MAPK and PKB, but not of ERK1/2, was diminished in RacT17N-transfected PASMC. Thrombin-induced TF mRNA and protein expression was attenuated in PASMC transfected with vectors encoding either a catalytically inactive PAK1 mutant or a kinase-deficient PKB mutant. Inhibition of p38MAPK and of phosphatidyl-inositol-3 kinase, but not of ERK1/2, diminished TF mRNA and protein levels in response to thrombin. These findings indicate that activation of a Rac and PAK1-dependent pathway is centrally involved in the regulation of TF expression and TF-dependent procoagulant activity in response to thrombin in human pulmonary artery smooth muscle cells and point towards a critical role of Rac and PAK1 in promoting the prothrombotic state and pulmonary vascular remodeling in pulmonary hypertension.

Shear Stress-Induced Elastase Fragmentation of Laminin to E8 Prevents Endothelial Cell Proliferation and Differentiation

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Activated metallo-proteases generate matrix fragments that may contribute to vascular remodeling by affecting proliferation and differentiation. We investigated whether shear stress contributes to the release of elastase and whether elastase fragments of laminin influence endothelial cell (EC) proliferation and differentiation. EC were subjected to shear stress (20 dyn/cm^2 for 2 h) and their conditioned media were tested for elastase activity. Western blot analysis of matrix proteins was performed to identify proteolytic laminin fragments. In proliferation assays EC were plated on either collagen I, fibronectin, laminin I, or its fragments P1 (pepsin) or E8 (elastase) stimulated by bFGF and measured with MTT. Apoptosis assays were performed in monitoring annexin V binding. Oxygen radical production was quantified by cytochrome reduction. Angiogenesis assays (sproutings) were performed using bFGF-stimulated aortic rings embedded in Matrigel, which was in part supplemented with laminin fragment E8. Shear stress enhanced elastase activity in conditioned media from shear stress cells ($p < 0.05$, $n = 8$). This went along with an increased occurrence of the laminin fragment E8 within the matrix. Seeding EC on E8 resulted in a reduced proliferation ($p < 0.05$, $n = 8$), increased apoptosis rate ($n = 4$, $p < 0.05$), and increased O_2^- production. Aortic ring sprouting was significantly inhibited by E8, which was revoked by application of radical scavengers SOD or catalase. Upon shear stress EC release elastase, which degrades laminin I into fragment E8, which, in contrast to intact laminin I, inhibits proliferation, induces apoptosis and oxygen radical production, and inhibits angiogenesis. These results indicate that during shear stress the extracellular matrix is altered post synthesis, a mechanism which might be part of a negative feedback loop to limit shear stress-induced proliferation and differentiation of endothelial cells in adaptive vascular remodeling.

Up-Regulation of Vascular Endothelial Growth Factor in Skeletal Muscles of Mice during Prazosin-Induced Angiogenesis

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Treatment of rodents with the α_1-adrenergic blocker prazosin is an effective method to induce angiogenesis in skeletal muscle. We isolated endothelial cells (ECs)-enriched fractions by precipitation with BS-I lectin-coated magnetic beads from skeletal muscles (tibialis anterior, extensor digitorum longus and rectus femoris) of C57Bl6-mice as well as NOS-1 (n-NOS, present in muscle fibers) and NOS-3 (e-NOS, present in endothelial cells) knockout mice, fed with prazosin for up to four days. Western blot analysis showed a continuous increase of VEGF in ECs of C57Bl6 mice treated with prazosin for one, two or four days while angiopoietin-2 and TIE-2 expression was unchanged. This upregulation of VEGF was also seen in ECs from NOS-1 knockout mice but not from NOS-3 knockout mice. We conclude that the muscle fiber and endothelial NOS/NO-systems differentially influence the VEGF expression in endothelial cells of skeletal muscles during angiogenesis.

Selective Photothermolysis of Blood Vessels Using Indocyanine Green and Laser Irradiation

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By careful selection of wavelength, pulse duration, and intensity of a laser vessels can be destroyed selectively. So far, a wavelength of 585 nm is preferred because it maximizes the selective absorption by hemoglobin. However, tissue penetration of light at this wavelength is limited. Therefore, to improve therapeutic outcome of thicker lesions indocyanine green (ICG) was used absorbing at 805 nm allowing deeper light penetration. The dorsal skinfold chamber model in hamsters ($n = 48$) was used for monitoring the vascular effects of ICG (0, 2 or 4 mg/kg b.w.; ICG-Pulsion, Munich, Germany) and diode laser irradiation ($\lambda = 805$ nm; pulse duration: 3, 10 or 30 ms; fluence: 3.2, 10.6 or 32 J/cm^2). Diameters of vessels marked with FITC-dextran (MW 150,000) were measured using intravital fluorescence microscopy prior to and 15 min, 1 h and 24 h following irradiation. At the end of each experiment histology was taken and tissue sections were stained by H&E, NBTC or CD31. A mathematical model calculates the intravascular temperature increase with respect to vessel diameter. Irradiation with a pulse duration of 30 ms and without ICG did hardly reduce the number of perfused vessels. After irradiation using 2 mg/kg b.w. ICG and 10 ms the number of perfused blood vessels decreased 1 h after irradiation but recovered to baseline at 24 h. Increasing the pulse duration up to 30 ms reduced the number of perfused vessel at 24 h by approx. 30% at a diameter of 8–10 µm. Using a higher concentration of ICG (4 mg/kg b.w.) and a pulse duration of 30 ms reduced the number of perfused vessels maximally by 53% at a diameter of 7–8 µm. The selective vascular damage was

confirmed by histology and immunohistochemistry 24 h after irradiation. This study shows for the first time the selective destruction of blood vessels following intravenous injection of ICG and subsequent irradiation with a pulsed diode laser.

Effect of Perfluorochemical (Oxycyte) Treatment on Permanent Focal Cerebral Ischemia in Rats

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Oxycyte is a new perfluorocarbon-based emulsion to function as an artificial oxygen carrier. The present study was conducted to evaluate the neuroprotective effect of Oxycyte treatment in focal cerebral ischemia in male rats (Sprague-Dawley, 270–350 g body weight). A permanent middle cerebral artery (MCA) occlusion was induced by positioning an intravascular suture. Animals received an intravenous infusion of 10 ml/kg body weight Oxycyte or isotonic saline at the time of MCA occlusion and were allowed to breath pure oxygen (normobaric hyperoxygenation, nbHO) or normal air. Animals were sacrificed 8 h after MCA occlusion and the necrotic volume was calculated from silver nitrate-stained serial slices. In addition, immunohistochemical staining for nitrotyrosine was performed. Ischemic volume was 353 ± 21 mm^3 in saline-nbHO animals and 370 ± 31 mm^3 in Oxycyte/air-treated animals as compared to 335 ± 36 mm^3 in control (saline-air) animals. However, animals treated with Oxycyte breathing pure oxygen had a significantly reduced infarct size (309 ± 44 mm^3) as compared to saline-treated rats breathing oxygen and animals receiving Oxycyte emulsion breathing air. Immunhistochemical staining of nitrotyrosine as an indirect measure of peroxynitrite formation yielded intense-staining in the ischemic region, mainly around capillaries. The intensity of peroxynitrite staining was most pronounced in saline-treated animals breathing air whereas it appeared markedly suppressed with Oxycyte treatment. Therefore, treatment with Oxycyte and nbHO commencing early after onset of brain ischemia may be of therapeutic efficacy to salvage ischemic brain tissue.

Cerivastatin Enhances the Expression of CYP 2c and Increases Protein Tyrosine Nitration in Coronary Artery Endothelial Cells

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HMG-CoA reductase inhibitors (statins) exert beneficial effects on endothelial function, which are partially attributable to an increased expression of the endothelial nitric oxide (NO) synthase (eNOS). Since statins are metabolized by cytochrome P450 2C (CYP 2C) enzymes in the liver and CYP 2C in endothelial cells has been identified as an endothelium-derived hyperpolarizing factor (EDHF) synthase as well as an important source of reactive oxygen species (ROS) in porcine coronary arteries (PCA), we determined the effects of fluvastatin and cerivastatin on NO- and EDHF-mediated relaxation, CYP 2C expression and radical formation in PCA. Incubation of coronary segments with cerivastatin (0.1 µmol/l, 18 h) or fluvastatin (1 µmol/l, 18 h) increased the bradykinin-induced NO- and prostacyclin-independent relaxations. This increase in EDHF-mediated relaxation correlated with an increase in CYP 2C mRNA (1.8 ± 0.2 vs. control) and protein. Under the same conditions, the expression of eNOS protein was also increased (cerivastatin 1.48 ± 0.17, fluvastatin 1.14 ± 0.05 vs. control), while the NO-mediated relaxation was not improved by pretreatment (18 h) of the vessels with either statin. In histochemical analyses, ROS production and the tyrosine nitration of vascular proteins were found to be markedly increased in statin-treated PCA. These increases were inhibited by co-administration of the specific CYP 2C9 inhibitor, sulfaphenazole (10 µmol/l). In summary, our data indicate that in PCA, cerivastatin and fluvastatin increase the expression of eNOS and the EDHF synthase CYP 2C, thus in coronary endothelial cells expressing a higher amount of both enzymes, the enhanced generation of NO and superoxide anions, and subsequently of peroxynitrite, can compromise the beneficial effects of statins on vascular reactivity.

Acetylcholine Induces Endothelial Cell Hyperpolarization along the Arteriolar Wall in vivo – Evidence from Measurements with Microelectrodes in the Microcirculation

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The endothelium releases different autacoids in response to acetylcholine (ACh) stimulation. Presumably, a hyperpolarizing factor (EDHF) contributes significantly to its dilator effect. Because locally induced hyperpolarizations can spread via gap junctions along the vascular wall, EDHF may be important for coordination of vascular behaviour. In order to verify hyperpolarizations induced by ACh in the murine microcirculation, we measured membrane potentials (MP) of vascular cells with sharp microelectrodes. The cremaster muscle of mice was prepared for in vivo microscopy and arterioles were carefully dissected from the surrounding skeletal muscle over a short distance of approximately 800 µm. In these areas, vascular cells were penetrated with microelectrodes (resistance: 60 MΩ). Criteria for successful penetration were a sharp signal deflection, a stable potential and low resistance over the membrane. Cells were identified by their shape, visualized by dye staining via the pipette. The resting MP of endothelial cells (EC) varied from −45 to −68 mV. Upon local stimulation of the arteriole with ACh via an ejection pipette, EC hyperpolarized regularly. The amount of hyperpolarization was dependent on the initial MP and on ACh concentration. Minimally achieved MP ranged from −65 to −72 mV, the maximal amplitude was 20 mV. In addition, the duration was lengthened with higher ACh concentrations. Hyperpolarizations were also found if the stimulation pipette was placed at a distant upstream site along the arteriole (up to 800 µm). Initial experiments indicate that hyperpolarizations at distant locations are attenuated in amplitude and duration after incubation of the stimulation site with charybdotoxin,

a blocker of Ca^{2+}-dependent K^+ channels. These experiments show that MP measurements in vivo are feasible with electrodes. The data demonstrates that ACh hyperpolarizes EC and that the hyperpolarization is not confined to the ACh application site.

NO-, but Not Acetylcholine-Induced Arteriolar Dilations Are Impaired in cGMP-Dependent Protein Kinase-Deficient Mice

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The NO/cGMP signal cascade plays an essential role in relaxing conducting arteries under resting conditions as well as upon endothelial stimulation with acetylcholine (ACh). One effector of cGMP is the cGMP-dependent protein kinase I (cGKI). Its role in dilations in resistance vessels is, however, not clear. We studied the contribution of GKI in dilations induced by NO or ACh by intravital microscopy and blood pressure measurements in mice deficient for GKI ($GK^{-/-}$). Diameter of arterioles in the cremaster muscle of wildtype (wt) and $GK^{-/-}$ mice were measured before and after superfusion of ACh or SNP. In addition, arterial pressure was measured in awake mice via a catheter inserted into the carotid artery. ACh and SNP were injected via this catheter as a bolus at different doses. SNP induced concentration-dependent dilations in wt mice (10 μ*M*: 71 ± 4%), but only small responses in $GK^{-/-}$ (22 ± 3%). In contrast, dilations upon ACh were similar in both genotypes (10 μ*M*: 86 ± 3 vs. 82 ± 4%). Mean arterial pressures in awake mice were not different between genotypes (wt: 118 ± 4, $GK^{-/-}$: 111 ± 6 mm Hg). While arterial bolus injection of SNP induced dose-dependent pressure drops in wt mice (15 nmol: by 51 ± 6 mm Hg), in $GK^{-/-}$ animals pressure decreased slightly at high doses only (15 nmol: by 9 ± 8 mm Hg). However, the ACh-induced decrease in pressure was not attenuated in $GK^{-/-}$ animals (5 nmol: wt 58 ± 5, $GK^{-/-}$ 70 ± 5 mm Hg). This data demonstrates that GKI is indeed the effector of the NO/cGMP pathway to induce arteriolar dilation. Because ACh dilations were unaffected, we conclude that the NO/cGMP/GK I pathway is not important for ACh-induced dilations in the microcirculation. The contrasting results reported previously for larger arteries stimulated with ACh suggest that the importance of endothelium-dependent hyperpolarization, as a mechanism for dilator responses upon ACh, increases with decreased vessel size.

Effect of EDHF and External 11,12-EETs on the Membrane Potential of Human Platelets

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EDHF from endothelial cells (EC) should not only affect adjacent vascular smooth muscle cells but also blood constituents. We therefore investigated whether EDHF could affect the membrane potential of platelets. Membrane potential (MP) was assessed by the fluorescent dye $DiBac_4(3)$ (0.5 μ*M*; FACS analysis). Human umbilical vein endothelial cells (HUVEC) were grown in presence of β-naphtoflavone (0.1 μ*M*) and nifedipin (3 μ*M*; both enhancing CYP2C9 expression), and stimulated with bradykinin (BK; 0.1 μ*M*) in the presence of L-NA (100 μ*M*) and indomethacin (30 μ*M*) to release EDHF. Sulfaphenazole (SPH; 30 μ*M*) an inhibitor of CYP2C8/9 and the K^+ channel inhibitors charybdotoxin (CHTX), iberiotoxin (IBTX), or apamin (APA; 500 n*M* each) were used as functional EDHF blockers. Resting MP of washed human platelets was –58 ± 9 mV (n = 10). The cation ionophor gramicidin (0.1 μ*M*) induced a platelet depolarization (by 19 mV) whereas the potassium ionophor valinomycin caused hyperpolarization (by –10 mV). The supernatant of BK-stimulated HUVECs which expressed CYP2C8 but not CYP2C9 (rt-PCR) induced also platelet hyperpolarization which was inhibited by pretreatment of HUVEC with SPH or platelets with CHTX or IBTX but not APA. 11,12-epoxyeicosatrienoic acids (EET) are products of CYP2C8/9-arachidonic acid metabolism, which have been identified to be an EDHF in certain vessels. We therefore, assessed their effects on platelet function. 11,12-EET and to a lesser extent 8,9-EET (but not 14,15-EET) dose-dependently hyperpolarized platelets in a CHTX/IBTX inhibitable manner. 11,12-EETs also inhibited ADP-induced surface expression of platelet P-selectin (n = 6, $p < 0.05$), which was dependent on functioning platelet K_{Ca}-channels. We conclude that endothelial cells release a factor that hyperpolarizes platelets, which has similar properties as 11,12-EETs. EDHF/EET could be of importance for platelet adhesion molecule expression and thereby regulate platelet functions.

Cell-to-Cell Coupling via Cx37-Containing Gap Junctions Is Specifically Reduced by NO

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Cell-to-cell communication in endothelial and smooth muscle cells is mediated via gap junctions, which are formed by the connexins (Cx) 37, 40, 43 and 45. In the vascular system, Cx37 and Cx43 are supposed to play a major role in myoendothelial coupling and therefore their regulation and the following effect on the gap junction channel conductivity are of interest. As NO reduces the cell-to-cell coupling in endothelial cells, we analysed, whether Cx37 or Cx43 are specifically targeted. HeLa cells that do not have endogenous Cx were transfected with either Cx37 (HeLaCx37) or Cx43 (HeLaCx43). Gap junction communication (GJC) was analyzed by injecting a fluorescent dye (Alexa Fluor 488) into single cells, determining the number of stained cells 12 min after injection and normalizing them to the total number of cells in the microscopic field (% coupled cells). The GJC was not affected by the NO donor SNAP (2 μM) in HeLaCx43 cells. HeLaCx37 cells showed such a low basic coupling, that an effect of NO was hardly detectable. In contrast, in mixed cultures of Cx37 and Cx43 cells (identified by different membrane staining), 58 ± 9% of the cells were coupled under control conditions (after injection in HeLaCx37) and SNAP reduced this coupling by 80%; HeLaCx43 cells in the same dishes remained unaffected. To further analyze the pathways involved in the NO effect, we blocked the soluble guanylyl cyclase with ODQ (10 μ*M*) before incubating the

cells with SNAP. The inhibition of the cGMP-depending pathway reduced the NO effect by 50%. Pretreatment with the cGMP-elevating compounds CNP and ANP (1 μ*M*) did not affect the GJC in HeLaCx37 and HeLaCx43 cells. These results suggest, that NO acts partially via cGMP. To reveal the importance of other signalling pathways – like the activation of PKC or direct phosphorylation of Cx – further experiments have to be done.

Role of Tyrosine Kinase syk in Depolarisation-Induced Endothelial Superoxide Formation

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Chronic increases in transmural pressure reduce NO-mediated dilations in isolated resistance arteries. This may be partly due to enhanced rac/NADH oxidase-dependent superoxide (O_2^-) production by endothelial cells in response to membrane depolarisation. The aim of the present study was therefore to investigate whether an increase in transmural pressure leads to membrane depolarisation of endothelial cells in intact vessels. Furthermore, we investigated in human umbilical vein endothelial cells (HUVEC) whether depolarised cells released a factor stimulating O_2^- production and/or whether the non-receptor-dependent kinase syk that has been shown to activate the small G-protein rac in hematopoetic cells is involved in the depolarisation-induced signal cascade. Using the voltage-sensitive dye di-8-ANEPPS we showed in isolated hamster arteries (n = 4) that an increase in transmural pressure (from 45 to 100 mm Hg) caused an endothelial depolarisation. In bioassay experiments no transfer of a soluble factor from depolarised cells (TBA and Gramicidin) could be detected (DCF method). In contrast, the syk inhibitor Piceatannol (10 μ*M*) inhibited the depolarisation induced O_2^- formation in HUVEC (DCF method) (n = 12). In addition, HUVEC transfected with syk anti-sense oligonucleotides did not show anymore an increase in superoxide formation following depolarisation whereas cells transfected with scrambled oligonucleotides still responded (cytochrom C method). It is concluded that endothelial membrane depolarisation occurs during increases of vascular transmural pressure. This depolarisation initiates a signal cascade whereby syk is involved. Our results suggest a new role for this non-receptor-dependent kinase in controlling O_2^- formation in endothelial cells.

The gp91phox-Containing NADPH Oxidase Mediates Vascular Dysfunction in Renovascular Hypertension

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An NADPH oxidase is a major source of vascular superoxide anion ($^{\bullet}O_2^-$) production in the vasculature. We determined the role of this enzyme for vascular dysfunction in renovascular hypertension induced by the 2-kidney, 1-clip model (2K1C) in wild-type (WT) and NADPH oxidase knockout (gp91phox$^{-/-}$) mice. 4 weeks after operation, systolic blood pressure was significantly higher and clipped kidney to non-clipped kidney weight ratio was significantly reduced in 2K1C in comparison to sham-operated mice. 2K1C induced more severe hypertension in WT than in gp91phox$^{-/-}$ mice (158 ± 2 versus 149 ± 2 mm Hg; $p < 0.05$), while systolic blood pressure did not differ between the two sham groups. Acetylcholine (Ach)-induced endothelium-dependent relaxation in isolated aortic rings was slightly better in sham-operated gp91phox$^{-/-}$ than WT mice. In WT, but not gp91phox$^{-/-}$ mice, renal artery clip application induced a pronounced attenuation of endothelium-dependent relaxation, whereas endothelium-independent relaxation to sodium nitroprusside was identical in all groups. The reactive oxygen species (ROS) scavenger tiron selectively enhanced Ach-induced relaxation in aortae of WT but not of gp91phox$^{-/-}$ mice, and this effect was more pronounced in aortae from animals subjected to clip operation than in sham controls. The protein kinase C inhibitor Ro 31-8220, which blocks the activation of the NADPH oxidase, enhanced Ach-induced relaxation of aortic segments from clipped and sham-operated WT, but not from gp91phox$^{-/-}$ mice. In the presence of Ro 31-8220, relaxation curves from the four groups were virtually superimposable. These data indicate that the activation of a gp91phox-containing NADPH oxidase and the subsequent scavenging of nitric oxide by NADPH oxidase-derived $^{\bullet}O_2^-$ contributes to the development of renovascular hypertension and underlies endothelial dysfunction in the 2K1C model.

The Insulin-Induced Production of Platelet-Derived Vasodilator Is Regulated by Nitric Oxide

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Insulin-induced vasodilatation in healthy subjects is sensitive to nitric oxide synthase inhibitors. However, insulin is unable to relax isolated arteries or to activate eNOS in cultured endothelial cells. Since eNOS is expressed in platelets, it is tempting to speculate that the insulin-induced vasodilatation observed in vivo can be attributed to the release of NO from platelets. Insulin failed to relax endothelium-intact rings of porcine coronary artery precontracted with U46619. However, the supernatant from insulin-stimulated human platelets induced complete relaxation. The supernatant from unstimulated platelets, or from platelets incubated with N^{ω}nitro-*L*-arginine and insulin, did not induce relaxation. Inhibition of either the AMP-dependent kinase (AMPK) or the phosphatidylinositol 3-kinase (PI3K) significantly reduced the insulin-induced, platelet-dependent relaxation. Similarly, incubation of platelets but not of detector rings with either a soluble guanylyl cyclase (sGC) or protein kinase G (PKG) inhibitor inhibited the insulin-induced, platelet-dependent relaxation. Relaxation was however abolished by adenosine receptor antagonists and was significantly inhibited by addition of adenosine deaminase to the supernatant. These results suggest that insulin stimulates the activation of eNOS in platelets by activating AMPK and Akt and that NO, via the activation of the sGC and PKG, stimulates the release of adenosine from platelets. The release of adenosine from platelets may account for the vasodilator response to insulin.

Ca-Independent Endothelial and Smooth Muscle Signaling Pathways of Wall Shear Stress (WSS) and Pressure in Arterioles

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We hypothesized that changes in endothelial $[Ca^{2+}]i$ are different in agonist- and WSS-induced release of NO. In isolated gracilis muscle arterioles ACh and increases in WSS (by increasing flow) elicited NO-mediated dilations. In diameter-clamped arterioles ACh caused substantial increases in endothelial Ca-fluorescence ratio (ERCa, max. 43 ± 5%) that was significantly greater than increases in ERCa (max. ~ 10%) to increases in WSS. A23187 substantially increased ERCa (max. 38 ± 5%) and elicited NO-mediated dilation (max. 45 ± 7%). Intraluminal tyrosine kinase inhibitor genistein had no effect on dilations to ACh or NO donor SNP, but eliminated those to WSS. Thus in arteriolar endothelium NO synthesis is activated by WSS without a substantial increase in $[Ca^{2+}]i$, most likely by activation of tyrosine kinase pathways, whereas NO release by ACh and A23187 is associated with substantial increases in $[Ca^{2+}]i$. Next, we hypothesized that the roles of smooth muscle PKC and MAP kinases in the modulation of agonist- and pressure-induced arteriolar constrictions are different. Constrictions to pressure, NE or Ang II were assessed in the absence or presence of chelerythrine (CH), PD98058 (PD) and SB203580 (SB, inhibitors of PKC, p42/44 and p38 MAP kinase pathways, respectively). Responses to NE and Ang II were reduced by CH (by ~ 90%) but not by SB, whereas PD decreased only constrictions to AII (by ~ 60%). Pressure-induced increases in wall tension elicited constrictions (max. 50%) that were abolished by CH. PD and SB decreased the myogenic tone (by 20% and 60%, respectively) and reduced the sensitivity of the myogenic mechanism to wall tension. Thus, PKC is involved both in myogenic and agonist-induced constrictions; PD-sensitive p42/44 MAP kinases modulate both wall tension-dependent and AII-induced constrictions, whereas SB-sensitive p38 MAP kinase pathway seems to be specific to wall tension. Collectively, Ca-independent pathways are importantly involved in the arteriolar mechanotransduction.

Regulation of Dynamic Actin Structures in Podocytes by CD2AP, a Crucial Protein for Glomerular Filtration

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The actin-based interdigitating foot processes of podocytes establish the essential portion of the filtration barrier in glomerular capillaries. Mice lacking the 80-kDa CD2-associated protein (CD2AP) develop progressive renal failure that starts with podocyte foot process effacement and increased protein permeability of glomerular capillaries by unknown mechanisms. We recently described that CD2AP is located in the interdigitating foot processes in situ and in cytoplasmic actin spots of cultured podocytes, staining positive for the actin nucleating Arp2/3 complex and cortactin [Welsch et al., Am J Physiol Renal Physiol 281: F769–777, 2001]. In the present study we provide evidence that CD2AP directly colocalizes with cortactin in actin spots. Moreover, CD2AP as well as cortactin and the Arp2/3 complex are components of ring-like structures (RiLiS) composed of clustered actin spots. By time-lapse microscopy, we found that RiLiS are dynamic actin structures, expanding with a mean velocity of 1.2 µm/min (n = 6). Motility of RiLiS could be stopped with jasplakinolide (1 µ*M*), an inhibitor of actin depolymerization, demonstrating the functional importance of actin turn-over in RiLiS. RiLiS formation was about 6-fold reduced by dislocalizing the Arp2/3 complex with a microinjected C-terminal WASP domain as compared to control peptide injection (n = 297 and 319 injected cells). In contrast, microinjection of an antibody directed against the C-terminal half of CD2AP resulted in formation of about 2-fold more RiLiS as compared to control IgG injection (n = 285 and 289 injected cells). Our results suggest that CD2AP, being part of a defined protein complex, negatively regulates the reorganization of the actin cytoskeleton in podocytes. Dysregulation of the actin cytoskeleton in podocytes may account for the loss of barrier function of glomerular capillaries in mice lacking CD2AP.

Altered Cerebrovascular Reactivity after Traumatic Brain Injury

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The aim was to characterize the alterations of cerebrovascular reactivity following global traumatic brain injury (TBI) in rats. In male SD rats anesthetized with chloralhydrate (360 mg/100 g i.p.) TBI of moderate degree was induced with a weight drop approach. Rats were killed after 24 or 48 h and ring segments prepared from the basilar artery (BA) and the middle cerebral artery (MCA) for measurement of isometric force. Vasoactive stimuli studied are i) contraction by incubation in 124 m*M* K^+ Krebs solution or cumulative applied endothelin (ET)-1, ii) endothelium-dependent relaxation by acetylcholine (ACh, BA), bradykinin (Bk, MCA) and the selective ET_B-receptor agonist, sarafotoxin-6c (S6c); all mediated by release of nitric oxide (NO), and iii) endothelium-independent relaxation by sodium nitroprusside (SNP) and 8-bromoguanosine cyclic monophosphate (cGMP). Relaxation was tested after pharmacological precontraction with serotonin (5-HT, BA) and the thromboxane mimetic U46619 (MCA). Contraction due to 124 m*M* K^+ Krebs (reference contraction) was markedly decreased after trauma (MCA/BA: control, 2.1 ± 0.8/5.1 ± 2.0 mN; 48 h after TBI, 1.3 ± 0.4*/3.2 ± 1.9* mN; * $p < 0.001$) as were contractions with 5-HT and U46619. Relaxation upon ACh and Bk was significantly enhanced whereas endothelium-independent relaxation induced by SNP and cGMP were not altered. However, ET-1-induced contraction was significantly enhanced after TBI (MCA/BA; % reference: control, 104 ± 40/95 ± 17; TBI, 166 ± 34*/142 ± 52*; * $p < 0.05$) while S6c-induced relaxation was shifted to higher concentrations (MCA/BA; pD_2: control, 9.6 ± 0.5/10.0 ± 0.7; TBI, 7.9 ± 0.4*/8.2 ± 0.7*; * $p < 0.001$). Global TBI results in differential alterations of cerebrovascular reactivity. Contraction to a variety of stimuli was decreased, while NO-mediated relaxation appeared preserved or even enhanced. In contrast, ET-1-induced contraction was increased, probably due to a decrease of ET_B-receptor-mediated relaxation.

Fibroblast Growth Factor-2 (FGF-2) Determines Cerebral Blood Flow Autoregulation

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The ability of cerebral arterioles to dilate under hypotensive stress and maintain cerebral blood flow (CBF) represents an integral component of the CBF autoregulation. Although disturbances of CBF autoregulation represent a common clinical problem following brain injury, the mechanisms underlying its regulation are only incompletely understood. Recently, FGF-2 has been demonstrated to play an important role in the regulation of extracerebral microvascular reactivity and in particular the arteriolar dilatory capacity. In order to test the hypothesis that FGF-2 is also involved in CBF autoregulation, we prepared closed cranial windows in FGF-2 wild type (FGF-2 +/+) and knockout (FGF-2 –/–) mice. For monitoring of mean arterial blood pressure catheters were implanted into the right carotid artery of the animals. Mean arterial blood pressure was gradually decreased from 100 to 20 mm Hg by venous pooling in a hypobaric chamber while the diameter of pial arterioles (PA) was visualized by intravital microscopy. Our study revealed a significantly impaired autoregulatory response in FGF-2 –/– mice with a maximum dilation of PA during hypotension of 20 ± 10% versus 45 ± 7% in controls. Interestingly, an intra-arterial substitution of FGF-2 failed to reconstitute the disturbed CBF autoregulation in FGF-2 –/– mice (PA dilation by 25 ± 15%) suggesting more complex disturbances within the FGF-2 –/– microcirculation. In order to further elucidate this defect in microvascular regulation, we compared the cerebral vessel morphology and expression of NOS isoforms between FGF-2 –/– and FGF-2 +/+ mice. However, so far our analyses have failed to show significant differences which might explain how FGF-2 contributes to CBF autoregulation. In summary, our study suggests a novel function for FGF-2, i.e. the involvement of CBF autoregulation. Future studies will have to focus on the underlying mechansims and the clinical relevance of these results.

Effects of Thoracic Epidural Anesthesia on Intestinal Capillary Perfusion during Endotoxemia

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Objective: Endotoxemia leads to intestinal arteriolar vasoconstriction and hypoperfusion [Crit Care Med 24:1233–1237, 1996]. Thoracic epidural anesthesia (TEA) has recently been shown to increase mucosal perfusion in ileum of healthy rats [Anesthesiology 93:844–851, 2000]. In this study we analyzed the effect of TEA on intestinal perfusion in rats during endotoxemia. **Methods:** Anesthetized rats were equipped with a thoracic epidural catheter. Epidural infusion was initiated by a bolus of 30 µl lidocaine 2% (TEA) or normal saline (CTRL), followed by a continuous infusion of 100 µl/h. Endotoxemia was induced by intravenous infusion of *E. coli* LPS (1.5 mg/kg/h). Capillary density of both, the mucosa and the muscularis as well as villus arteriole diameters were determined. The density of non-perfused capillaries as percentage of total capillary density was assessed. Immunohistochemistry with tyrosine-hydroxylase antibodies was performed to show sympathetic innervation of the terminal ileum in untreated rats. Statistics: ANOVA and Student's t test. Values as median and 25/75th percentile. **Results:** After 60 min of endotoxemia, non-perfused capillaries increased in the mucosa of both groups (CTRL: 25% [15/38%]), TEA: 18% [6/36%]). After 120 min non-perfused capillaries further increased (CTRL: 42% [24/46%]; TEA: 46% [22/58%]). In the muscularis, a significant rise in non-perfused capillaries only appeared in CTRL after 120 min of endotoxemia (19% [8/55%]). The TEA group did not show any perfusion deficits. Villus arteriole constricted in the CTRL group whereas it dilated in the TEA group. Immunohistochemistry revealed sympathetic innervation of vessels in muscularis and submucosa, but not in the mucosa. **Conclusion:** During Endotoxemia TEA prevents constriction of villus arteriole and perfusion deficits in the muscularis but not in the mucosa. This may be explained by the scarce sympathetic innervation of the mucosa in rats' terminal ileum.

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