Endothelial preconditioning by transient oxidative stress reduces inflammatory responses of cultured endothelial cells to TNF-α

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ABSTRACT Brief episodes of ischemia can render an organ resistant to subsequent severe ischemia. This 'ischemic preconditioning' is ascribed to various mechanisms, including oxidative stress. We investigated whether preconditioning exists on an endothelial level. Human umbilical vein endothelial cells (HUVECs) were transiently confronted with oxidative stress (1 mM H_2O_2, 5 min). Adhesion molecules ICAM-1 and E-selectin and release of cytokines IL-6 and IL-8 to subsequent stimulation with TNF-α (2.5 ng/ml, 4 h) were measured (flow cytometry and immunoassay), as were nuclear translocation of the transcription factor NFκB (Western blotting, confocal microscopy) and redox status of HUVECs (quantification of glutathione by HPLC). TNF-α elevated IL-6 in the cell supernatant from 8.8 ± 1 to 41 ± 3 pg/ml and IL-8 from 0.5 ± 0.03 to 3 ± 0.2 ng/ml. ICAM-1 was increased threefold and E-selectin rose eightfold. Oxidative stress (decrease of glutathione by 50%) reduced post-TNF-α levels of IL-6 to 14 ± 3 and IL-8 to 1 ± 0.2; the rise of ICAM-1 was completely blocked and E-selectin was only doubled. The anti-inflammatory effects of preconditioning via oxidative stress were paralleled by reduction of the translocation of NFκB on stimulation with TNF-α, and antagonized by the intracellular radical scavenger N-acetylcysteine. 'Anti-inflammatory preconditioning' of endothelial cells by oxidative stress may account for the inhibitory effects of preconditioning on leukocyte adhesion *in vivo*.— Zahler, S., Kupatt, C., Becker, B. F. Endothelial preconditioning by transient oxidative stress reduces inflammatory responses of cultured endothelial cells to TNF-α. *FASEB J.* 14, 555–564 (2000)

Key Words: cytokine · adhesion molecule · NFκB · glutathione

REPERFUSION OF PREVIOUSLY ischemic tissue paradoxically leads to an increase of tissue damage and dysfunction (1, 2). In the light of yearly rising numbers of cardiac interventions invoking transient ischemia (PTCA, thrombolysis, coronary bypass grafting, valve replacement, transplantation, CPB), the incidence of reperfusion, and thus reperfusion injury, gains ever-increasing relevance. Reperfusion is restricted mostly to clinical settings, and therefore is a planned intervention. Accordingly, awareness has grown that patients might benefit substantially from a deeper knowledge of the mechanisms underlying reperfusion injury, since this should lead to subsequent development of protective strategies.

To date, the most powerful tool for experimentally reducing infarct size after ischemia and reperfusion is the so-called 'ischemic preconditioning'. This phenomenon was first described by Murry and co-workers (3), who have shown that brief, transient episodes of ischemia 'precondition' myocardium to become more resistant against a subsequent severe ischemic insult. Since then the research interest in this effect has grown constantly, and a variety of potential mechanisms have been proposed: adrenergic stimulation (4), activation of adenosine receptors (5), opening of K_{ATP} channels (6), induction of heat shock proteins (7), and induction of oxidative stress (8), just to mention those that are established best. However, the effector cells of preconditioning are only poorly defined. Apart from parenchymal cells, especially cells of the vascular wall seem of interest, because reperfusion injury is, at least in part, a misguided inflammatory response (9). In this sense, ischemic preconditioning has previously been shown to reduce adhesion of leukocytes to postischemically reperfused vessels (10).

The proinflammatory mediator tumor necrosis factor α (TNF-α) plays a key role during ischemia and reperfusion (11, 12), modulating cell adhesion molecules, cytokines, and chemokines. We have therefore investigated whether the responses of endothelial cells to TNF-α can be modulated by a protocol mimicking ischemic preconditioning. The stimulus chosen was that of brief redox stress imposed by transient application of hydrogen peroxide. Oxidative stress has been shown to occur during preconditioning (13) and to be mandatory for the

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success of preconditioning in some models (14, 15). This also seemed of broader interest because the modulation of cellular redox status has recently turned out to be a major signal for inflammatory reactions (16, 17).

Preconditioning at first seemed to be a temporally limited phenomenon, being acutely effective only within periods of less than 1 h. Recently, however, a 'second window of protection' has been identified, occurring some 24 h after the preconditioning period (7, 18). Thus, we also tested whether an anti-inflammatory effect still pertains after a 24 h lag phase between preconditioning and inflammatory stimulus still allows.

The inflammatory parameters measured in cultures of human umbilical vein endothelium were the expression of the adhesion molecules ICAM-1, E-selectin, and P-selectin and secretion of the cytokines interleukin 6 (IL-6) and IL-8. The redox status of the cells was monitored by separate measurement of the intracellular concentration of reduced glutathione (GSH). Application of the GSH precursor N-acetyl-cysteine served to manipulate the intracellular redox status. Potential effects of the experimental protocols on cell apoptosis and necrosis were investigated by measurement of annexin-V binding and of lactate dehydrogenase (LDH) release. Involvement of the transcription factor NFκB (nuclear factor κB), which has been shown to be both redox sensitive (19) and to participate in the regulation of the inflammatory parameters listed above, was also studied.

MATERIALS AND METHODS

Endothelial cell culture

Human umbilical vein endothelial cells (HUVECs) were isolated from fresh umbilical cords according to the method of Jaffe (20). Briefly, the umbilical vein was cannulated, rinsed with phosphate buffer, and filled with collagenase A (1 mg/ml) for 5 min at 37°C. Endothelial cells were eluted with medium 199, centrifuged, washed, pelleted again, and then seeded with endothelial cell growth medium (Promocell, Heidelberg, Germany) into T 25 culture flasks. After reaching confluence, the primary cultures were detached from the flask with trypsin and transferred into 24-well culture dishes coated with collagen A (Biochrom, Berlin, Germany). Experiments were performed 2 days after the cells had reattained confluence.

Flow cytometry

The expression levels of the adhesion molecules ICAM-1, E-selectin, and P-selectin on HUVECs, as well as the binding of annexin-V to these cells, were quantified by flow cytometry. Cell monolayers were washed with phosphate-buffered saline (PBS) without calcium and incubated with a trypsin solution (in the case of P-selectin, a collagenase solution, P-selectin being rapidly activated in response to trypsin) until the cells were detached from the culture dish. The cells were treated

with CellFix (Becton Dickinson, Heidelberg, Germany) and passed through a nylon net (70 μm mesh). The cells were pelleted by centrifugation, resuspended in CellWash (Becton Dickinson), labeled with the respective antibodies, washed, and measured on a FACScan flow cytometer (Becton Dickinson). The antibodies MCA675PE, MCA883F, and MCA796PE (all Serotec, Kidlington, U.K.) were used to detect ICAM-1, E-selectin, and P-selectin, respectively. Data analysis was performed with Lysis II software (Becton Dickinson). The median of the specific fluorescence intensity was used as a marker for expression of the respective epitope; nonspecific fluorescence was detected by using isotype-matched nonbinding antibodies (Serotec) and subtracted. Binding of annexin-V to HUVECs was determined with an apoptosis detection kit (PharMingen, Heidelberg, Germany) containing FITC-labeled annexin-V, propidium iodide, and a calcium-rich binding buffer. The staining protocol complied with the manufacturer's instructions. Median fluorescence intensity of FITC on propidium iodide negative (i.e., living) cells was used as a measure of apoptosis.

Photometric tests

Levels of the cytokines IL-6 and IL-8 were determined in cell culture supernatants with commercially available ELISAs (Endogen, Woburn, Mass.). To determine IL-8, the samples were diluted 1:100. Sample aliquots of 50 μl were used per well of the test plate in all cases; staining and measurement were performed as proscribed by the manufacturer. Briefly, the ELISA plates were incubated with the standards or samples for 60 min. Unbound cytokine was removed by washing three times, and a second antibody (labeled enzymatically for photometric detection) was added. After further incubation and removal of unbound antibody by washing, the plates were analyzed photometrically with a microplate reader (Dynatech, Guernsey, U.K.) at 450 nm. As an indicator of cellular death, release of the enzyme LDH into the supernatant was also measured photometrically at 490 nm (CytoTox96 assay, Promega, Mannheim, Germany).

Measurement of GSH

After removal of medium, HUVECs were lysed with 400 μl/well of a stopping solution (64 mM N-ethylmaleimide in 0.5 M perchloric acid) to prevent autoxidation of GSH. Concentrations in the lysate are termed intracellular, no attempt having been made to correct this to the real intracellular volume. GSH was analyzed directly by high-performance liquid chromatography (HPLC) as the NEM adduct at a wavelength of 202 nm. Two hundred microliters of the respective sample were applied to a 5 μm C-18 nucleosil column (Macherey and Nagel, Düren, Germany); 25 mM $NH_4H_2PO_4$ containing 1.2% methanol served as eluent. At a flow of 1 ml/min, the retention time was ~13 min.

Western blot analysis

HUVECs were lysed in a reducing Triton lysis buffer (Tris 1 mM, NaCl 50 mM, Triton 1×, sodium vanadate 5 mM, NaF 50 mM, Na pyrophosphate 30 mM, DTT 1 mM, PMSF 1 mM, leupeptin 10 μM, pepstatin 10 μM). Nuclear and cytosolic fractions were separated by centrifugation (10,000 g for 5 s), the membrane fractions were removed by 15 min centrifugation at 10,000 g (4°C). The protein concentrations of the lysates (nuclear and cytosolic) were determined with a detergent compatible assay (BCA, Pierce, Rockford, Ill.). Aliquots (40 μg) of protein were separated on a 12% polyacylamide gel, transferred to a nitrocellulose membrane (Hybond-en-

hanced chemiluminescence, Amersham, Braunschweig, Germany), blocked with buffer containing 5% nonfat dry milk, and incubated with 2 μg/ml of primary antibody (anti-p65 subunit of NFκB or anti-IκB, both from Santa Cruz Biotechnology, Santa Cruz, Calif.). After washing four times with buffer containing 1% nonfat dry milk, the membranes were exposed to 0.2 μg/ml of secondary antibody, labeled with horseradish peroxidase (Santa Cruz). The membranes were then incubated with an enhanced chemiluminescence kit (Pierce) for 1 min and exposed to an X-ray film. The films were analyzed with a video system (GelDoc 1000, Bio-Rad, Hercules, Calif.) and optical density of the bands was calculated with MolecularAnalyst software (Bio-Rad). Due to considerable interindividual variability, values are expressed as percentual changes vs. controls (100%). Analogous experiments were performed with the cytoplasmatic fractions to control the purity of the nuclear and cytoplasma preparation, respectively.

Confocal microscopy

To determine the subcellular localization of NFκB, stimulated HUVECs were fixed with buffered formaldehyde (3%) and subsequently permeabilized by submersion in 0.2% Triton X-100 in PBS for 2 min. The samples were rinsed three times with PBS, blocked with 0.2% BSA in PBS for 15 min, and incubated with the primary antibody against p65 (Santa Cruz) for 45 min. After four washings, the secondary antibody (FITC labeled, Santa Cruz) was added and samples were incubated for 30 min at room temperature. After four final washing steps, the cells were covered with mounting medium (Sigma, Eching, Germany) and a coverslip before inspection with a confocal microscope (LSM 410 Invert, Zeiss, Jena, Germany).

Experimental protocols

Flow charts of the experimental protocols *A–C* are depicted in **Fig. 1**. In preconditioning experiments, medium was removed from confluent HUVECs and replaced by PBS containing 1 mM H_2O_2 for 5 min at 37°C. Afterward, the supernatant was removed and the cells were covered with standard medium again. The cells were then incubated with TNF-α (2.5 ng/ml) for 4 h, after which supernatant was sampled for measurement of IL-6, IL-8, and LDH. Cells were detached and treated

for flow cytometric analysis of ICAM-1, E-selectin, P-selectin, and annexin-V binding (Fig. 1*A*). Time matched controls were treated with neither H_2O_2 nor TNF-α. Further experimental groups consisted of cells that had been treated with H_2O_2 (5 min) or TNF-α (4 h) alone. In protocol *B*, these experiments were repeated in cells that had been pretreated with the intracellular radical scavenger N-acetylcysteine (NAC, 1 mM) for 30 min. After this incubation, the cells were washed to remove all extracellular NAC and then subjected to the aforementioned procedures. Some of the experiments of protocols *A* and *B* were terminated 5 min or 30 min after the H_2O_2 stimulus to allow for determination of intracellular GSH or for analysis of the subcellular localization of NFκB or IκB (Fig. 1). To investigate whether transient redox stress causes a second window of protection against stimulation with TNF-α, a 24 h interval was allowed between stimulation with H_2O_2 and TNF-α. During this time the cells were kept in the incubator with standard medium. Afterward, TNF-α was given for 4 h and the experiments were continued as described above (Fig. 1, protocol *C*).

Statistical procedures

Data are usually expressed as mean ± SE; *n* values are shown in Results. Statistical analysis was performed with 2-way ANOVA. One factor for group comparisons was treatment with TNF-α or not; the other factor was treatment with H_2O_2 or not. Pairwise multiple comparison tests were carried out using the Student-Newman-Keuls test. Differences between groups were considered significant for $P \leq 0.05$.

RESULTS

Expression of adhesion molecules

HUVECs showed constitutive expression of ICAM-1 on a low level (mean fluorescence intensity 3.9±0.3 arbitrary units, **Fig. 2**). Short incubation with H_2O_2 (1 mM for 5 min) did not alter ICAM-1 expression within 4 h. In contrast, stimulation with TNF-α (2.5 ng/ml for 4 h) elevated ICAM-1 ~threefold
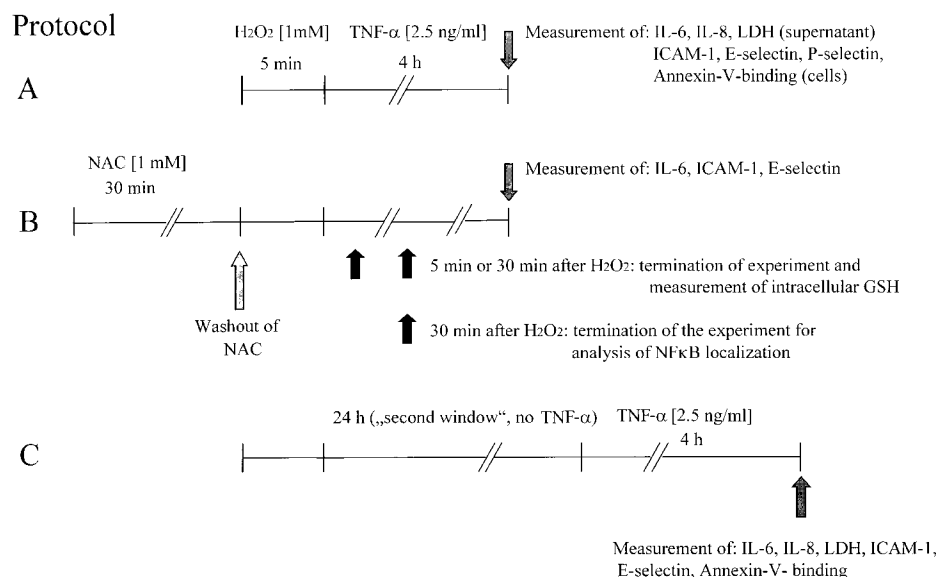
Figure 1. Flow diagram of the various experimental protocols. *A)* Standard protocol, *B)* preincubation with N-acetylcysteine (NAC), *C)* 24 h latency between H_2O_2 and TNF-α treatment (second window of protection).
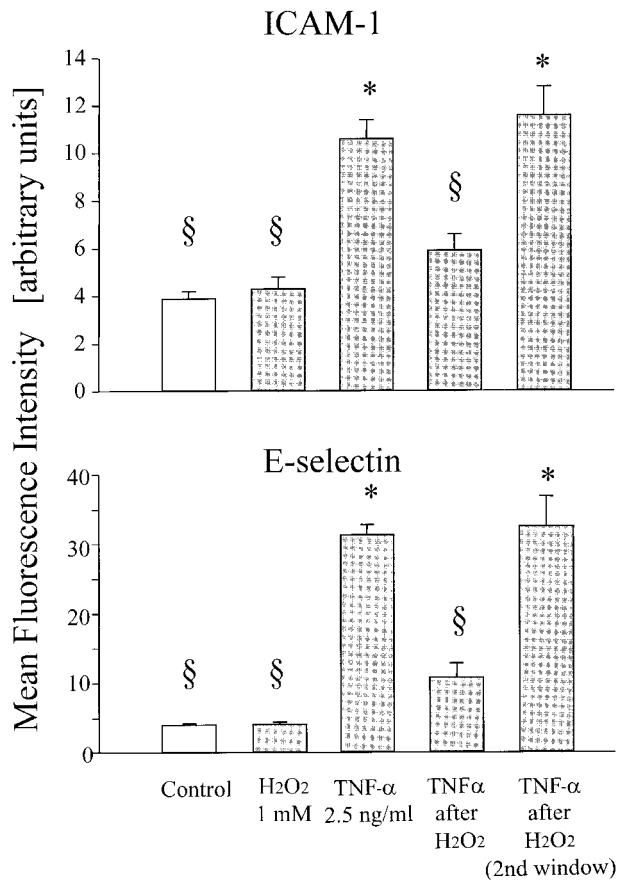
ICAM-1

E-selectin

Figure 2. Expression of the adhesion molecules ICAM-1 (upper panel) and E-selectin (lower panel) on HUVECs, measured by flow cytometry. Controls: $n=19$ different cultures. H_2O_2: $n=14$, TNF-α: $n=31$, TNF-α after H_2O_2: $n=19$, 2nd window: $n=6$. Means $\pm$ SE; *significantly different vs. control, §significantly different vs. TNFα, $P \leq 0.05$.

$(10.6\pm0.8$, Fig. 2). Pretreatment with H_2O_2 strongly reduced the effect of TNF-α (5.9 ± 0.7, Fig. 2). This action was not observed when stimulation with TNF-α occurred 24 h after treatment with H_2O_2 (Fig. 2). Preincubation of the HUVECs with the intracellular radical scavenger NAC (1 mM, 30 min) did not alter constitutive ICAM-1 levels or the response to TNF-α (4.0 ± 0.7 and 12.9 ± 1.1, respectively, Fig. 4), whereas the inhibitory effect of H_2O_2 on TNF-α-dependent ICAM-1 expression was abolished (9.9 ± 2.5).

Expression of the adhesion molecule E-selectin was regulated in an analogous way. Basal expression (4.0 ± 0.2, Fig. 2) was unaltered by H_2O_2 alone, whereas TNF-α led to an eightfold increase (31 ± 3, Fig. 2). This was dramatically infringed by pretreatment with H_2O_2 (10.8 ± 2, Fig. 2). Again, there was no second window after 24 h. NAC did not influence the constitutive E-selectin level (3.5 ± 0.3) or the effect of TNF-α (32.2 ± 4.8, Fig. 4). However, the blocking action of H_2O_2 was nearly neutralized by NAC (27.5 ± 3.7).

The presence of P-selectin behaved in a different

way: H_2O_2, as well as TNF-α or the combination of both stimuli, elevated basal expression from 4.15 ± 0.13 to 5.38 ± 0.17, 5.14 ± 0.16, and 5.88 ± 0.34, respectively (**Table 1**).

Secretion of cytokines

Cytokines IL-6 and IL-8 were both constitutively secreted by HUVECs (8.9 ± 1.4 and 450.4 ± 36 pg/ml, respectively, **Fig. 3**). H_2O_2 alone caused no changes, whereas TNF-α increased the levels of both cytokines substantially to 40.6 ± 3.3 for IL-6 and 2960 ± 192 pg/ml for IL-8 (Fig. 3). These increases were mitigated by application of H_2O_2 prior to TNF-α (14.4 ± 2.9 and 1015 ± 159 pg/ml, resp., not significant vs. control). When TNF-α was applied with a latency of 24 h after H_2O_2 treatment, this inhibitory effect was only marginal and not significant. Preincubation of the cells with NAC decreased constitutive levels of IL-6 release to 3.2 ± 1.5 pg/ml, but did not affect the increase mediated by TNF-α (45.6 ± 2 pg/ml, **Fig. 4**). The loss of efficacy of TNF-α after H_2O_2 was less pronounced under the influence of NAC (25 ± 4 pg/ml, Fig. 4). IL-8 release was not determined in this series.

Levels of GSH in cell lysates

Intracellular GSH levels, as determined in cell lysates, are illustrated in **Fig. 5**. Control levels of GSH in HUVEC lysates were 0.48 ± 0.03 μM. This value was reduced by half (0.25 ± 0.01 μM) 5 min after stimulation with H_2O_2, whereas TNF-α (5 min) had no effect (0.53 ± 0.03 μM). The loss of GSH due to H_2O_2 was not aggravated or reduced by additional application of TNF-α (0.26 ± 0.03 μM). Pretreatment with NAC doubled the constitutive GSH level (0.85 ± 0.04 μM). Though H_2O_2 also caused a reduction of GSH in this protocol ($0,44\pm0.03$ μM), the resulting concentration did not differ from the resting level in untreated cells. Again, TNF-α had no effect, either alone (0.75 ± 0.03 μM) or in combination with H_2O_2 (0.38 ± 0.02 μM). At 30 min after stimulation, GSH levels were still slightly reduced in all the H_2O_2-treated cells (Fig. 5), but this decrease

TABLE 1. *Flow cytometric evaluation of P-selectin expression on HUVECs 4 h after stimulation[a]*

Experimental condition	P-selectin specific fluorescence (arbitrary units)
Control	4.15 ± 0.13
H_2O_2 (1 mM, 5 min)	5.39 ± 0.17*
TNFα (2.5 ng/ml)	5.14 ± 0.16*
TNFα after H_2O_2	5.88 ± 0.34*

^{a} $n = 6$ for all conditions; * significant vs. control ($P \leq 0.05$), means $\pm$ SD.
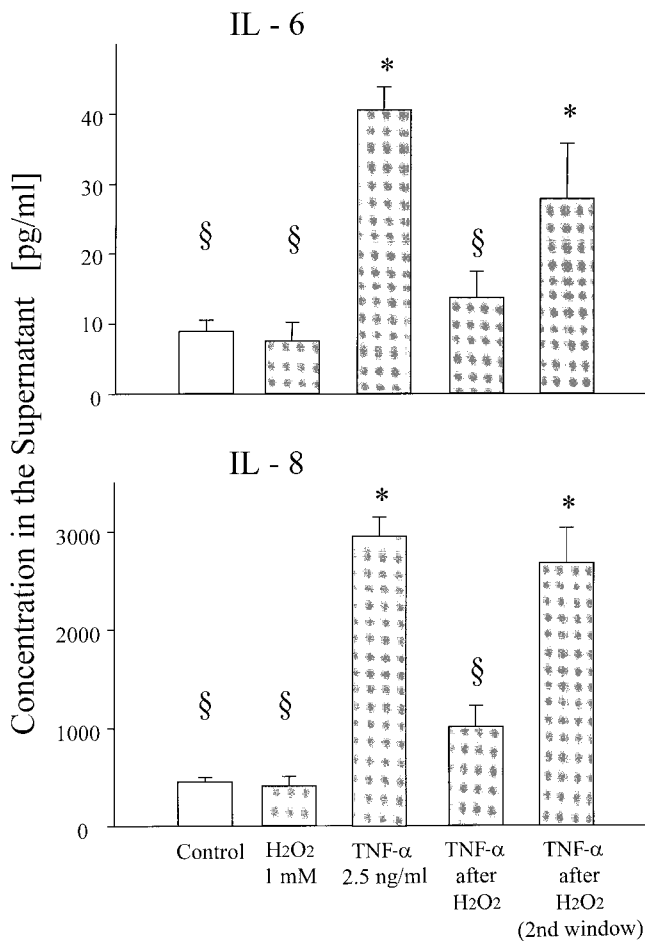
Figure 3. Concentrations of the cytokines IL-6 (upper panel) and IL-8 (lower panel) in the supernatant of HUVECs were measured by ELISA. Controls: $n=19$ cultures, H_2O_2: $n=14$, TNF-α: $n=31$, TNF-α after H_2O_2: $n=19$, 2nd window: $n=6$. Means ± SE; *significantly different from control, §significantly different vs. TNFα, $P \leq 0.05$.

did not reach statistical significance vs. controls and the TNF-α group.

Quantification of necrosis and apoptosis

To establish whether the observed effects on inflammatory parameters were perhaps caused by modulation of cell necrosis or apoptosis, LDH levels in the supernatant and annexin-V binding on the cells were quantified (**Fig. 6**). LDH in the supernatant of control cells amounted to 22.5 ± 1.1 mU/ml and did not change significantly in any group. Annexin-V binding was not altered by H_2O_2 either alone or in combination with TNF-α (Fig. 6). TNF-α concentration dependently increased annexin-V-specific fluorescence from 4.1 ± 0.2 units (control) to 7.4 ± 0.9 at 300 ng/ml (Fig. 6).

IκB and NFκB

IκB was consistently found in the cytoplasm of control HUVECs (defined as 100%). Addition of H_2O_2

or TNF-α caused severe reductions in IκB levels (31±9% and 53±14%, resp., **Table 2**). A combination of both stimuli did not show any interference or augmentation. IκB was absent from all nucleus preparations, which suggests good separation quality (data not shown).The p65 subunit of NFκB was detected in the cytoplasm of resting cells and, to a much lower extent, in the nuclei. This basal presence of p65 in the nucleus (set at 100%) was also to be seen in the immunohistochemical confocal images (**Fig. 7A**). Both H_2O_2 and TNF-α, separately caused increases of nuclear NFκB (190±52% and 253±115%, resp., Table 2 and Figs. 7A, B). However, the effect of TNF-α on translocation of NFκB was totally inhibited by pretreatment of cells with H_2O_2, as shown by Western blotting and confocal microscopy (Table 2 and Figs. 7A, B).
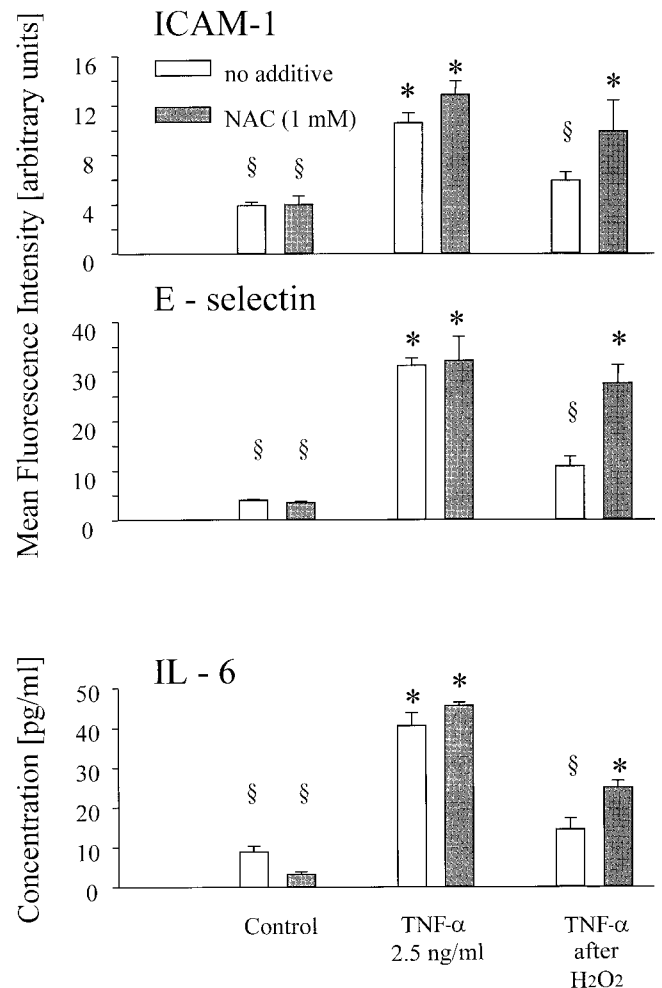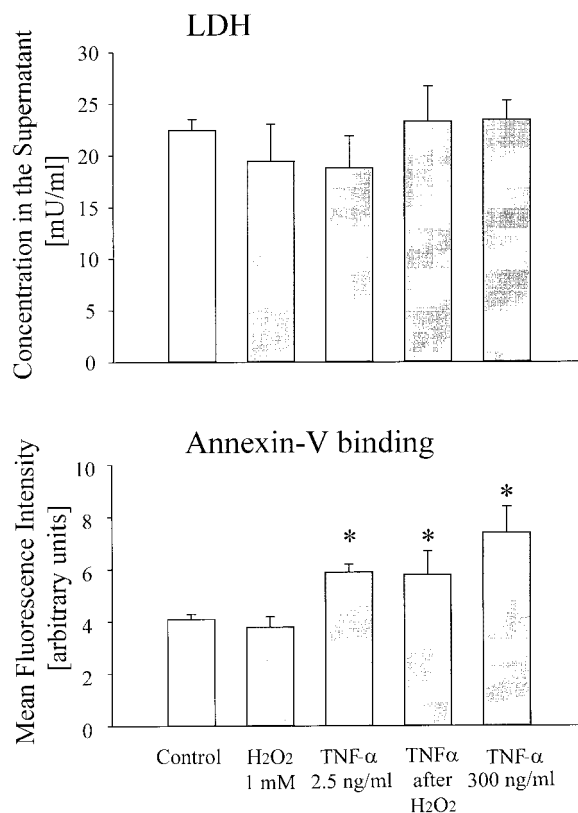

Figure 4. Effect of the intracellular radical scavenger NAC (N-acetylcysteine) on expression of ICAM-1 (upper panel) and E-selectin (middle panel), and on release of IL-6 (lower panel). $n=6$ cultures for all experimental groups. Means ± SE; *significantly different from respective control, §significantly different vs. respective TNF-α group, $P \leq 0.05$.

5 min after stimulation

30 min after stimulation
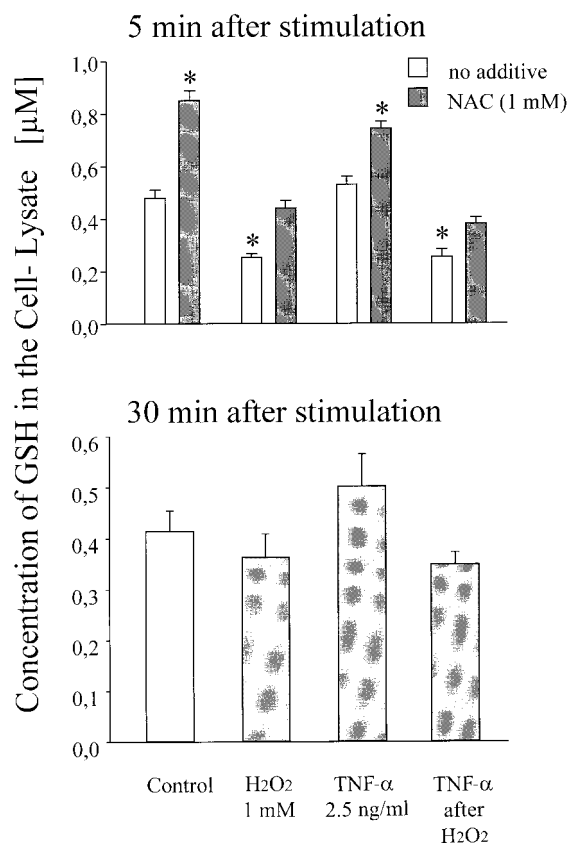
Figure 5. GSH levels in the lysates of HUVECs, 5 min (upper panel) and 30 min (lower panel) after initiation of different stimulation protocols. $n=6$ cultures for all conditions; means ± SE; *significantly different from control, $P \leq 0.05$.

LDH

Annexin-V binding

Figure 6. Upper panel: concentration of LDH in the supernatant of HUVECs incubated 4 h under various conditions. Lower panel: flow cytometric data on annexin-V binding to HUVECs after 4 h. Controls: $n=7$ cultures, H_2O_2 : $n=6$, TNF-α 2.5 ng/ml: $n=9$, TNF-α (2.5 ng/ml) after H_2O_2: $n=8$, TNF-α 300 ng/ml: $n=6$. Means ± SE; *significantly different from controls, $P \leq 0.05$.

DISCUSSION

Since its discovery in 1986 (3), the phenomenon of 'ischemic preconditioning' has aroused considerable interest. On the one hand, this is due to the fact that preconditioning is the most powerful tool known at present to reduce postischemic infarct size (21). On the other hand, the attention results from the growing clinical relevance and frequency of reperfusion scenarios. Knowledge of the underlying principles might help to design pharmacological interventions, keeping tissues at risk in a 'permanent state of preconditioning' (21). Accordingly, numerous studies have been performed to elucidate the mechanisms behind preconditioning, but the results have suggested nearly as many different mediators, including adenosine (5), acetylcholine (22), catecholamines (4), angiotensin II (23), bradykinin (24), nitric oxide (25), and reactive oxygen species (8). To make things even more complicated, the cellular targets of preconditioning seem to be heterogeneous. Though saline perfused isolated hearts or isolated cardiomyocytes can be preconditioned (15, 26), other reports suggest a microvascular component of preconditioning (27–29). This mainly concerns the reduction of postischemic inflammation or, more precisely, the mitigation of leukocyte adhesion in the

reperfused vascular bed that has been described after preconditioning (10, 30). Accordingly, it is tempting to speculate that the vascular endothelium, the interface between parenchymal tissue and blood cells, is involved in this phenomenon.

Oxidative stress has been shown to occur after preconditioning (13) and to be a mandatory factor of preconditioning at least in some models (14). Moreover, oxidative stress and changes in cellular redox status have turned out to be powerful modulators of intracellular signaling (19). For example, the expression of adhesion molecules and cytokines

TABLE 2. *Densitometric evaluation of Western blots for IκB in the cytosolic fraction and for NFκB in the nuclear fraction of HUVECs lysed 30 min after intervention*[a]

Experimental condition	IκB in the cytoplasm	NFκB in the nuclear fraction
Control	100%	100%[§]
H_2O_2 (1 mM, 5 min)	31 ± 9%*	190 ± 52%*
TNFα (2.5 ng/ml)	53 ± 14%*	253 ± 115%*
TNFα after H_2O_2	47 ± 13%*	113 ± 75%[§]

[a] $n = 3$ for all conditions; * significant vs. control, [§] significantly different vs. TNFα, $P \leq 0.05$, means ± SD.
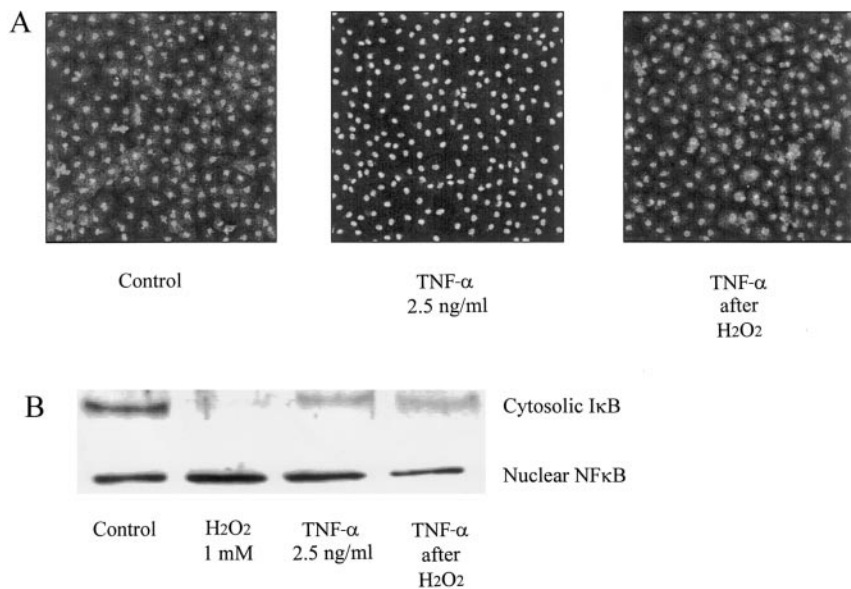
Figure 7. *A)* Immunofluorescent staining for the p65 subunit of NFκB. HUVECs were fixed in formalin 30 min after stimulation, permeabilized with Triton X-100, and stained with a p65 monoclonal antibody. Cells were inspected on a confocal microscope after incubation with an FITC-labeled secondary antibody. *B)* Representative Western blots for the expression of IκB in the cytosol and NFκB in the nuclear extract of HUVECs after different stimulation protocols. Quantitative densitometric evaluation is shown in Table 2.

by endothelial cells is demonstrably regulated by changes in intracellular redox status (16). Since leukocyte recruitment at the site of reperfusion is caused by exactly these endothelial factors, we endeavored to establish whether oxidative stress might elicit a kind of anti-inflammatory 'endothelial preconditioning'.

To this end, we subjected HUVECs to 5 min incubation with H_2O_2, a reagent that has turned out to be a reproducible and easily controllable tool for generating reversible intracellular redox stress (31). TNF-α was used as a subsequent inflammatory stimulus because this cytokine has been shown by us (12) and others (11) to be released during postischemic reperfusion and to be a key mediator or reperfusion injury. The inflammatory parameters we measured were chosen in order to cover the whole cascade of leukocyte adhesion to the vessel wall and subsequent transmigration: P-selectin, which is stored in endothelial vesicles and can be rapidly transferred into the cell membrane on stimulation, enabling rapid recruitment of leukocytes and rolling along the vessel wall (32). E-selectin and ICAM-1 allow for sustained rolling at later time points and for firm adhesion, respectively. Finally, by chemotactically activating the leukocytes, IL-6 and IL-8 mediate the transition from rolling to firm adhesion (sticking), and cause transmigration (for a review, see ref 33). P-selectin has previously been shown to be rapidly expressed (within minutes) on the surface of HUVECs after treatment with H_2O_2 (31). Four hours later, P-selectin on HUVECs was still elevated (Table 1), but to an extent that does not necessarily involve *de novo* synthesis. The same was true for TNF-α or the combination of both stimuli (Table 1). The small extent of these effects might be due to the fact that the promoter region of human P-selectin lacks κB sites, necessary to cause the pronounced up-regula-

tion of this adhesion molecule, e.g., in mice after stimulation with TNF-α (34). In contrast, as was to be expected, TNF-α caused dramatic increases of the expression of ICAM-1 and E-selectin (Fig. 2) and of the secretion of IL-6 and IL-8 (Fig. 3). Transient brief pretreatment with H_2O_2 inhibited all these inflammatory effects (Figs. 2 and 3), an action that could easily be interpreted as an endothelial preconditioning.

However, alternative explanations are also feasible. For instance, H_2O_2 can be a cytotoxic and proapoptotic stimulus, depending on concentration and incubation time. This is also true of TNF-α. So, if most of the cells were to be killed or made apoptotic when both stimuli are combined in the preconditioning protocol, a reduction in protein synthesis by these cells would not be surprising. Such relatively nonspecific effects, however, were ruled out: neither LDH release (a sign of necrosis) nor annexin-V binding (a marker of apoptosis) was elevated beyond the extent observed after stimulation with TNF-α alone (Fig. 6). The sensitivity of the assay seems appropriate, as TNF-α did cause a concentration-dependent increase of annexin-V binding when given alone (Fig. 6).

It could further be argued that the relatively high concentration of H_2O_2 (1 mM) might have caused nonspecific extracellular alterations of cellular structures (e.g., the TNF-α receptor). Thus, to differentiate between possible extracellular oxidative effects and intracellular actions, the intracellular scavenger NAC, a precursor of GSH was used. NAC has been reported to prevent the redox mediated part of TNF-α action (19). In the present paper, however, we chose a concentration and incubation time for NAC that did not influence the effects of TNF-α on ICAM-1, E-selectin, or IL-6 (Fig. 4). Nevertheless, the increase in intracellular GSH levels to ~200% of

control levels found after treatment with NAC (Fig. 5) demonstrated that NAC had been taken up and metabolized by the endothelial cells. In contrast to the indifferent behavior of NAC toward TNFα, the protective effect of transient H_2O_2 application was nearly abolished by pretreatment with NAC (Fig. 4). This finding suggests that the protective effect of preconditioning with H_2O_2 is caused by changes of intracellular redox status.

Indeed, using GSH as a marker of intracellular redox stress showed that application of H_2O_2 reduced GSH levels to ~50% of the control level (Fig. 5). This oxidative stress was transient, as it was detectable 5 min (but no longer 30 min) after treatment. After pretreatment with NAC, GSH levels also decreased on incubation with H_2O_2, but they did not fall below the concentrations seen in untreated control cells (Fig. 5). Thus, these cells presumably did not experience critical oxidative stress in the course of the experiment. Accordingly, it may be concluded that the preconditioning effect in our model is caused by brief, nonlethal intracellular redox stress.

The classical protocols of ischemic preconditioning (short periods of ischemia immediately followed by a more severe ischemic insult) have revealed the transient nature of this phenomenon. Some years ago, however, an additional time course of myocardial protection had been detected: ~24 h after the preconditioning process, there is a second phase of myocardial protection, which has been termed second window of protection (18). This kind of preconditioning has been ascribed to the delayed induction of cardioprotective mechanisms, like production of heat shock proteins (7). In this case, too, oxidative stress has been postulated as a key stimulus (26). However, when we extended our observations to a later time point, no influence on the inflammatory reactions of the endothelial cells was seen when 24 h lay between the applications of hydrogen peroxide and TNF-α (Figs. 2 and 3). The phenomenon of the second window of protection thus seems to be based on different mechanisms or it may not develop in HUVECs.

Most inflammatory parameters measured by us on HUVECs have been reported to be regulated by the transcription factor NFκB. Upon stimulation, NFκB is translocated from the cytosol into the nucleus. This process is enabled by previous phosphorylation, ubiquitination, and proteasomal degradation of the inhibiting factor IκB. Since translocation of NFκB has also been shown to be redox sensitive (19) and to occur early after cardiac ischemia and reperfusion (12, 35), we investigated whether alterations of NFκB-mediated signaling might be causal for the reduced inflammatory reactions of HUVECs subsequent to preconditioning by oxidative stress. The translocation of NFκB into the nuclear fraction differed between treatments: whereas H_2O_2 and TNF-α alone both caused translocation, sequential treatment (TNF-α after preconditioning with H_2O_2) was not additive and instead completely abolished the effects of the single stimuli (Figs. 7*A, B* and Table 2). The fact that P-selectin, an adhesion molecule that is not regulated by NFκB in human cells (34)—in contrast to the NFκB-dependent parameters ICAM-1, E-selectin, IL-6, and IL-8—did not respond to the preconditioning protocol (Table 1) further strengthens the hypothesis that NFκB is a selective target for the inhibitory effects of brief oxidative stress.

The identical decrease of IκB after treatment of HUVECs with either H_2O_2 alone, TNF-α alone, or TNF-α after H_2O_2 (Fig. 7*B* and Table 2) suggests that the inhibition of translocation in the latter intervention group is not due to a different degree of phosphorylation of IκB (Table 2). Alternative explanations for the decreased level of nuclear NFκB could be an infringed transport of NFκB to and into the nucleus, altered permeability of nuclear pores for NFκB, or a lower binding affinity between NFκB and the DNA. To our knowledge, the first two mechanisms have not been validated for NFκB. In contrast, a reduction of NFκB affinity toward its binding sites on DNA due to oxidative stress has been reported (36). However, in our model, no differences were observed in global oxidative stress (mirrored as GSH concentration) between treatment with H_2O_2 alone and with TNF-α after H_2O_2. This was the case both after 5 min or 30 min (Fig. 5), i.e., the time, when NFκB translocation was measured. Despite the absence of differences in global redox status between these two groups, it cannot be excluded that the redox state of specific thiols, which modulate TNF-α effects (17), might have been affected for this duration. A partial explanation of the anti-inflammatory actions of brief oxidative stress could be that NFκB down-regulates its own expression via a negative feedback loop (37). Indeed, we have found that overall content of NFκB in HUVECs pretreated with H_2O_2 (1 mM, 5 min) was reduced to ~60% of control cells 4 h after the treatment (data not shown). However, this change in NFκB expression cannot account for the rapid infringement of NFκB translocation 30 min after preconditioning.

The presence of NFκB in the nucleus is important but far from sufficient for the expression of ICAM-1, E-selectin, IL-6, and IL-8. Our data show, for instance, that redox stress in form of a brief pulse of H_2O_2 can cause translocation of NFκB without subsequent up-regulation of adhesion molecules or cytokines (Table 2; Figs. 2 and 3). TNF-α, in contrast, elicited NFκB translocation and higher expression levels of the proinflammatory parameters, but with-

out having induced oxidative stress. Thus, other (e.g., posttranscriptional) influences of the preconditioning protocol on inflammatory responses might occur in addition to the reduction of NFκB translocation. One feasible modulator of inflammatory reactions in this context might be nitric oxide, which on the one hand has been demonstrated to be involved in preconditioning in some models (25), and on the other has elicited protective actions during reperfusion (38).

In conclusion, we have shown for the first time that endothelial cells can be preconditioned by transient intracellular redox stress to reduce responsiveness to an inflammatory mediator. The finding that this phenomenon is related to infringed translocation of the transcription factor NFκB may enable novel approaches directed toward alleviating reperfusion damage. Though the underlying mechanisms are not resolved, endothelial preconditioning may well account for the anti-adhesive effects of ischemic preconditioning (10, 30) and its protective role in the microvasculature (27, 28, 29) *in vivo*. In a wider context, the intriguing finding that sequential stimuli can blunt each other with respect to translocation of NFκB might aid the further clarification of mechanisms involved in redox signaling. [FJ]

REFERENCES

1. Hearse, D. J. (1977) Reperfusion of the ischemic myocardium. *J. Mol. Cell. Cardiol.* **69,** 605–616

2. Ma, X.-L., Tsao, P. S., and Lefer, A. M. (1991) Antibody to CD-18 exerts endothelial and cardiac protective effects in myocardial ischemia and reperfusion. *J. Clin. Invest.* **88,** 1237–1243

3. Murry, C. E., Jennings, R. B., and Reimer, K. A. (1986) Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. *Circulation* **74,** 1124–1136

4. Banerjee, A., Locke Winter, C., Rogers, K. B., Mitchell, M. B., Brew, E. C., Cairns, C. B., Bensard, D. D., and Harken, A. H. (1993) Preconditioning against myocardial dysfunction after ischemia and reperfusion by an alpha 1-adrenergic mechanism. *Circ. Res.* **73,** 656–670

5. Hoshida, S., Kuzuya, T., Nishida, M., Yamashita, N., Oe, H., Hori, M., Kamada, T., and Tada, M. (1994) Adenosine blockade during reperfusion reverses the infarct limiting effect in preconditioned canine hearts. *Cardiovasc. Res.* **28,** 1083–1088

6. Schulz, R., Rose, J., and Heusch, G. (1994) Involvement of activation of ATP-dependent potassium channels in ischemic preconditioning in swine. *Am. J. Physiol.* **267,** H1341–H1352

7. Marber, M. S., Latchman, D. S., Walker, J. M., and Yellon, D. M. (1993) Cardiac stress protein elevation 24 h after brief ischemia or heat stress is associated with resistance to myocardial infarction. *Circulation* **88,** 1264–1272

8. Tritto, I., D'Andrea, D., Eramo, N., Scognamiglio, A., DeSimone, C., Violante, A., Esposito, A., Chiariello, M., and Ambrosio, G. (1997) Oxygen radicals can induce preconditioning in rabbit hearts. *Circ. Res.* **80,** 743–748

9. Chatelain, P., Latour, J. G., Tran, D., de-Lorgeril, M., Dupras, G., and Bourassa, M. (1987) Neutrophil accumulation in experimental myocardial infarcts: relation with extent of injury and effect of reperfusion. *Circulation* **75,** 1083–1090

10. Akimitsu, T., Gute, D. C., and Korthuis, R. J. (1996) Ischemic preconditioning attenuates postischemic leukocyte adhesion and emigration. *Am. J. Physiol.* **271,** H2052–H2059

11. Squadrito, F., Altavilla, D., Zingarelli, B., Ioculano, M., Calapai, G., Campo, G. M., Miceli, A., and Caputi, A. P. (1993) Tumor necrosis factor involvement in myocardial ischaemia-reperfusion injury. *Eur. J. Pharmacol.* **237,** 223–230

12. Kupatt, C., Habazettl, H., Goedecke, A., Wolf, D. A., Zahler, S., Boekstegers, P., Kelly, R. A., and Becker, B. F. (1999) Tumor necrosis factor-α contributes to ischemia- and reperfusion-induced endothelial activation in isolated hearts. *Circ. Res.* **84,** 392–400

13. Crestanello, J. A., Lingle, D. M., Kamelgard, J., Millili, J., and Whitman, G. J. R. (1996) Ischemic preconditioning decreases oxidative stress during reperfusion: a chemiluminescence study. *J. Surg. Res.* **65,** 53–58

14. Baines, C. P., Goto, M., and Downey, J. M. (1997) Oxygen radicals released during ischemic preconditioning contribute to cardioprotection in the rabbit myocardium. *J. Mol. Cell. Cardiol.* **29,** 207–216

15. Chen, W., Gabel, S., Steenbergen, C., and Murphy, E. (1995) A redox-based mechanism for cardioprotection induced by ischemic preconditioning in perfused rat heart. *Circ. Res.* **77,** 424–429

16. Kokura, S., Wolf, R. E., Yoshikawa, T., Granger, D. N., and Aw, T. Y. (1999) Molecular mechanisms of neutrophil-endothelial cell adhesion induced by redox imbalance. *Circ. Res.* **84,** 516–524

17. Friedrichs, B., Müller, C., and Brigelius-Flohé, R. (1998) Inhibition of tumor necrosis factor-α- and interleukin-1-induced endothelial E-selectin expression by thiol-modifying agents. *Arterioscler. Thromb. Vasc. Biol.* **18,** 1829–1837

18. Baxter, G. F., Marber, M. S., Patel, V. C., and Yellon, D. M. (1994) Adenosine receptor involvement in a delayed myocardial protection 24 h after ischemic preconditioning. *Circulation* **90,** 2993–3000

19. Schreck, R., Rieber, P., and Baeuerle, P. A. (1991) Reactive oxygen intermediates as apparently widely used messengers in the activation of the NFκB transcription factor and HIV-1. *EMBO J.* **10,** 2247–2258

20. Jaffe, E. A., Nachmann, R. L., Becker, C. G., and Minick, C. R. (1973) Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. *J. Clin. Invest.* **52,** 2745–2756

21. Yellon, D. M., Baxter, G. F., Garcia-Dorado, D., Heusch, G., and Sumeray, M. S. (1998) Ischaemic preconditioning: present position and future directions. *Cardiovasc. Res.* **37,** 21–33

22. Yao, Z., and Gross, G. J. (1993) Acetylcholine mimics ischemic preconditioning via glibenclamide-sensitive mechanism in dogs. *Am. J. Physiol.* **264,** H2221–H2225

23. Liu, Y., Tsuchida, A., Cohen, M. V., and Downey, J. M. (1995) Pretreatment with angiotensin II activates protein kinase C and limits myocardial infarction in isolated rabbit hearts. *J. Mol. Cell. Cardiol.* **27,** 883–892

24. Goto, M., Liu, Y., Yang, X. M., Ardell, J. L., Cohen, M. V., and Downey, J. M. (1995) Role of bradykinin in protection of ischemic preconditioning in rabbit hearts. *Circ. Res.* **77,** 611–621

25. Ferdinandy, P., Szilvassy, Z., Balogh, N., Csonka, C., Csont, T., Koltai, M., and Dux, L. (1996) Nitric oxide is involved in active preconditioning in isolated working rat hearts. *Ann. N.Y. Acad. Sci.* **793,** 489–493

26. Zhou, X., Zhai, X., and Ashraf, M. (1996) Direct evidence that initial oxidative stress triggered by preconditioning contributes to second window of protection by endogenous antioxidant enzyme in myocytes. *Circulation* **93,** 1177–1184

27. Jerome, S. N., Akimitsu, T., Gute, D. C., and Korthuis, R. J. (1995) Ischemic preconditioning attenuates capillary no-reflow induced by prolonged ischemia and reperfusion. *Am. J. Physiol.* **268,** H2063–H2067

28. DeFily, D. V., and Chilian, W. M. (1993) Preconditioning protects coronary arteriolar endothelium from ischemia-reperfusion injury. *Am. J. Physiol.* **265,** H700–H706

29. Richard, V., Kaeffer, N., Tron, C., and Thuillez, C. (1994) Ischemic preconditioning protects against coronary endothelial dysfunction induced by ischemia and reperfusion. *Circulation* **89,** 1254–1261

30. Kubes, P., Payne, D., and Ostrovsky, L. (1998) Preconditioning and adenosine in I/R-induced leukocyte-endothelial cell interactions. *Am. J. Physiol.* **274,** H1230–H1238

31. Zahler, S., Kupatt, C., Möbert, J., Becker, B. F., and Gerlach, E. (1997) Effects of ACE-inhibition on redox status and expression of P-selectin of endothelial cells subjected to oxidative stress. *J. Mol. Cell. Cardiol.* **29,** 2953–2960

32. Geng, J-G., Bevilacqua, M. P., Moore, K. L., McIntyre, T. M., Prescott, S. M., Kim, J. M., Bliss, G. A., Zimmerman, G. A., and McEver, R. P. (1990) Rapid neutrophil adhesion to activated endothelium by GMP-140. *Nature (London)* **343,** 757–760

33. Jordan, J. E., Zhao, Z-Q., and Vinten-Johansen, J. (1999) The role of neutrophils in myocardial ischemia-reperfusion injury. *Cardiovasc. Res.* **43,** 860–878

34. Pan, J., Xia, L., and McEver, R. P. (1998) Comparison of promoters for the murine and human P-selectin genes suggest species-specific and conserved mechanisms for transcriptional regulation in endothelial cells. *J. Biol. Chem.* **273,** 10058–10067

35. Li, C., Browder, W., and Kao, R. L. (1999) Early activation of transcription factor NFκB during ischemia in perfused rat heart. *Am. J. Physiol.* **276,** H543–H552

36. Hayashi, T., Ueno, Y., and Okamoto, T. (1993) Oxidoreductive regulation of nuclear factor κ B. *J. Biol. Chem.* **268,** 11380–11388

37. Lombardi, L., Ciana, P., Cappellini, C., Trecca, D., Guerrini, L., Migliazza, A., Maiolo, A. T., and Neri, A. (1995) Structural and functional characterization of the promoter regions of the NFκB2 gene. *Nucleic Acids Res.* **23,** 2328–2336

38. Siegfried, M. R., Erhardt, J., Rider, T., Ma, X-L., and Lefer, A. M. (1992) Cardioprotection and attenuation of endothelial dysfunction by organic nitric oxide donors in myocardial ischemia-reperfusion. *J. Pharmacol. Exp. Ther.* **260,** 668–675