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# Dexamethasone-Induced Expression of Endothelial Mitogen-Activated Protein Kinase Phosphatase-1 Involves Activation of the Transcription Factors Activator Protein-1 and 3′,5′-Cyclic Adenosine 5′-Monophosphate Response Element-Binding Protein and the Generation of Reactive Oxygen Species

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**We have recently identified the MAPK phosphatase (MKP)-1 as a novel mediator of the antiinflammatory properties of glucocorticoids (dexamethasone) in the human endothelium. However, nothing is as yet known about the signaling pathways responsible for the up-regulation of MKP-1 by dexamethasone in endothelial cells. Knowledge of the molecular basis of this new alternative way of glucocorticoid action could facilitate the identification of new antiinflammatory drug targets. Thus, the aim of our study was to elucidate the underlying molecular mechanisms. Using Western blot analysis, we found that dexamethasone rapidly activates ERK, c-jun N-terminal kinase (JNK), and p38 MAPK in human umbilical vein endothelial cells. By applying the kinase inhibitors PD98059 (MAPK kinase-1) and SP600125 (JNK), ERK and JNK were shown to be crucial for the induction of MKP-1. Using EMSA and a decoy oligonucleotide approach, the transcription factors activator protein-1 (activated by ERK and JNK) and cAMP response element-binding protein (activated by ERK) were found to be involved in the up-regulation of MKP-1 by dexamethasone. Interestingly, dexamethasone induces the generation of reactive oxygen species (measured by dihydrofluorescein assay), which participate in the signaling process by triggering JNK activation. Our work elucidates a novel alternative mechanism for transducing antiinflammatory effects of glucocorticoids in the human endothelium. Thus, our study adds valuable information to the efforts made to find new antiinflammatory principles utilized by glucocorticoids. This might help to gain new therapeutic options to limit glucocorticoid side effects and to overcome resistance. (*Endocrinology* 149: 3635–3642, 2008)**

SINCE THEIR INTRODUCTION in the 1950s, glucocorticoids, such as dexamethasone (Dex), have become valuable and indispensable drugs used for the treatment of a plethora of diseases with an inflammatory component, for instance chronic obstructive pulmonary disorders, rheumatoid arthritis, chronic inflammatory bowel diseases, or allergies. Glucocorticoids work by binding to their cytosolic glucocorticoid receptor (GR), which upon activation translocates to the nucleus. There, it modulates gene transcription by binding to glucocorticoid response elements in the respective target genes. Although the receptor activates some antiinflammatory genes (*e.g.* lipocortin-1), the antiinflammatory properties of glucocorticoids are thought to be predominantly mediated via a second pathway: The receptor, without binding to the DNA, inhibits proinflammatory transcription factors, such as nuclear factor-κB or activator protein-1 (AP-1), by a direct protein-protein interaction known as transrepression (1).

Besides these well-established concepts of action, additional antiinflammatory mechanisms of glucocorticoids have been proposed, especially cross-talk between glucocorticoids and MAPK signaling pathways (2). In recent years, MAPK phosphatase-1 (MKP-1), the first described member of the family of dual-specificity phosphatases, has emerged as a novel glucocorticoid-induced gene (3). In this context, we recently identified MKP-1 as a mediator of the antiinflammatory action of Dex in the human vascular endothelium (4). The endothelium is a fundamental component of the vascular system. In addition to its important regulatory functions regarding vascular permeability, blood pressure, and coagulation, the endothelium is crucially involved in inflammatory response processes. The extravasation of leukocytes from the blood to the sites of inflammation, tightly controlled by endothelial cells, is of great importance in this context. Adhesion molecules, like E-selectin, are necessary for the first steps in extravasation, *i.e.* the adherence of leukocytes to the endothelium, and are strongly expressed in inflammation-activated endothelial cells. In the model of TNF-α-exposed human endothelial cells, we have previously demonstrated that an increase of MKP-1 protein is responsible for the nuclear factor-κB-independent reduction of E-selectin expression caused by Dex at 1 nM. The Dex-induced up-reg-

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Abbreviations: AP-1, Activator protein-1; CREB, cAMP response element-binding protein; Dex, dexamethasone; JNK, c-jun N-terminal kinase; MEK1, MAPK kinase-1; MKP-1, MAPK phosphatase-1; NAC, *N*-acetyl-L-cysteine; PI3K, phosphatidylinositol 3-kinase; ROS, reactive oxygen species.

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ulation of MKP-1 was shown to inhibit TNF-α-activated p38 MAPK, which is responsible for the TNF-α-evoked increase of E-selectin (4).

In general, MKP-1 has increasingly been recognized as an important negative regulator of inflammatory processes (5–8) and as a gene up-regulated by glucocorticoids (3). The concept of MKP-1 as a crucial mediator of the antiinflammatory effects of glucocorticoids was recently verified *in vivo* (9). Nevertheless, the precise mechanisms that glucocorticoids use to regulate MKP-1 are as yet unknown (3). Therefore, the aim of this study was to clarify the molecular signaling pathways that are responsible for the increased expression of MKP-1 protein caused by Dex in the human vascular endothelium.

## Materials and Methods

### *Reagents*

The synthetic glucocorticoid dexamethasone [(11β,16α)-9-fluoro-11,17,21-trihydroxy-16-methylpregna-1,4-diene-3,20-dione], the antioxidants tiron (4,5-dihydroxy-1,3-benzenedisulfonic acid disodium salt) and *N*-acetyl-L-cysteine (NAC), and the phosphatidylinositol 3-kinase (PI3K) inhibitor wortmannin (KY 12420) were from Sigma-Aldrich (Taufkirchen, Germany). The MAPK kinase-1 (MEK1) inhibitor PD98059 (2′-amino-3′-methoxy-flavone) was from Cell Signaling/New England Biolabs (Frankfurt am Main, Germany). The c-jun N-terminal kinase (JNK) inhibitor SP600125 [anthra(1,9-cd)pyrazol-6(2H)-one] and the p38 MAPK inhibitor SB203580 [4-(4-fluorophenyl)-2-(4-methyl-sulfinylphenyl)-5-(4-pyridyl)1H-imidazole] were from Calbiochem/EMD Biosciences/Merck (Darmstadt, Germany).

### *Cell culture*

Human umbilical vein endothelial cells were prepared by digestion of umbilical veins with 0.1 g/liter collagenase A (Roche, Mannheim, Germany) as previously described (10) and cultured in endothelial cell growth medium (Promocell, Heidelberg, Germany) containing 10% heat-inactivated fetal calf serum (Biochrom, Berlin, Germany). Cells of passage 3 were used in all experiments and were routinely tested for mycoplasma contamination with the PCR detection kit VenorGeM (Minerva Biolabs, Berlin, Germany). Before treatment, cells were starved overnight in a steroid-free medium consisting of phenol red-free DMEM (Bio-Whittaker/Cambrex, Verviers, Belgium) supplemented with 20% heat-inactivated, charcoal-stripped fetal calf serum (Biochrom).

### *Immunoblotting*

For Western blot analysis, cells were grown in six-well plates until confluence and were treated as indicated in the respective figure legend. Western blot analysis was performed as described previously (10). Anti-MKP-1 rabbit polyclonal antibody (dilution 1:1000) was from Santa Cruz (Heidelberg, Germany). Anti-phospho-p44/42 (ERK) MAPK (Thr202/Tyr204) mouse monoclonal (E10) antibody (dilution 1:2000), anti-p44/42 (ERK) MAPK rabbit polyclonal antibody (dilution 1:1000), and anti-phospho-SAPK/JNK (Thr183/Tyr185) rabbit monoclonal (98F2) antibody (dilution 1:1000) were from Cell Signaling/New England Biolabs. Horseradish peroxidase-conjugated goat antirabbit antibody was from Dianova (Hamburg, Germany), and horseradish peroxidase-conjugated goat antimouse antibody was from Biozol (Eching, Germany). For densitometric analysis, films developed with an AGFA Curix 60 (AGFA, Cologne, Germany) were analyzed by the Kodak 1D software version 3.5.4 (Eastman Kodak, Rochester, NY). Regarding the densitometric analysis of JNK and ERK, the JNK1/p-JNK1 and ERK2/p-ERK2 bands were used for quantification.

### *EMSA*

Cells were grown in six-well plates or 60-mm dishes until confluence and treated as indicated in the respective figure legend. Nuclear extracts were prepared and EMSA was performed as described previously (11). AP-1 consensus oligonucleotides (5′-CGCTTGATGAGTCAGCCGGAA-3′) and cAMP response element-binding protein (CREB) consensus oligonucleotides (5′-AGAGATTGCCTGACGTCAGAGAGCTAG-3′) were from Promega (Mannheim, Germany). For densitometric analysis, the OptiQuant software version 4.00 (PerkinElmer, Rodgau, Germany) was used.

### *AP-1 and CREB decoy experiments*

Cells were grown in six-well plates until approximately 80% confluence and were transfected with decoy or scrambled decoy phosphorothioate oligonucleotides by using the SuperFect transfection reagent (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Experiments were performed 3 h after transfection. AP-1 decoy (5′-cgcttGATGACTCAGCcggaa-3′, lowercase letters indicate phosphorothioate backbone), AP-1 scrambled decoy (5′-cgcttGATGACTTGGCcggaa-3′), CREB decoy (5′-tgacgTCATGACGTCATGAcgtca-3′), and CREB scrambled decoy (5′-ctagcTAGCTAGCTAGCTAgctag-3′) were from biomers.net (Ulm, Germany).

### *Detection of reactive oxygen species (ROS)*

Cells were grown in 24-well plates until confluence and treated as indicated in the respective figure legend. ROS generation was detected as described previously (12). Briefly, cells were loaded with 20 μM dihydrofluorescein diacetate (Molecular Probes/Invitrogen, Karlsruhe, Germany) for 20 min. Fluorescence measurements were made 30 min after treatment. Data were calculated as percent increase of fluorescence values of untreated cells.

### *Statistical analysis*

The number of independently performed experiments (n) is stated in the respective figure legend. One representative image is shown. Bar graph data are expressed as mean ± SEM. Statistical analysis was performed with GraphPad Prism software version 3.03 (GraphPad, San Diego, CA). To compare three or more groups, one-way ANOVA followed by Tukey's *post hoc* test was used. To compare two groups, Student's *t* test was used.

## Results

As previously reported by our group, Dex induces the expression of MKP-1 in human endothelial cells. Maximal MKP-1 protein expression was observed after 60 min at a concentration of 1 nM Dex (4). Therefore, this time point and concentration were chosen for all following experiments regarding the induction of MKP-1 by Dex.

### *Dex treatment leads to an activation of ERK, JNK, and p38 MAPK*

Activated MAPKs up-regulate MKP-1 as a negative feedback mechanism. Therefore, we hypothesized that Dex activates these kinases. To judge their activity, the phosphorylation status at Thr202/Tyr204 (ERK), Thr183/Tyr185 (JNK), and Thr180/Tyr182 (p38 MAPK) was measured using Western blot analysis. Indeed, we found that all three MAPKs, ERK (Fig. 1A), JNK (Fig. 1B), and p38 MAPK (Fig. 1C), are rapidly (within 5–15 min) activated by Dex. This activation is transient and starts disappearing after 60 min.

### *ERK and JNK play a crucial role in the induction of MKP-1 by Dex, whereas no involvement of p38 MAPK or PI3K could be demonstrated*

To test whether and which MAPK is in fact responsible for the Dex-induced up-regulation of MKP-1, pharmacological

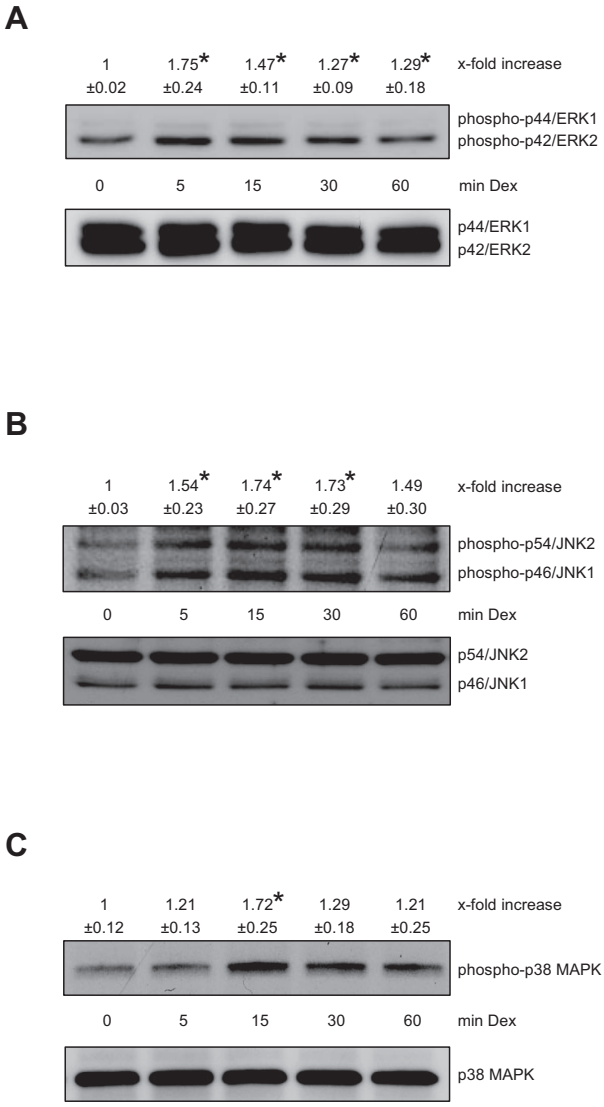


FIG. 1. Dex treatment time-dependently activates ERK, JNK, and p38 MAPK. A, Dex activates ERK (p42/p44) (n = 4); B, Dex activates JNK (p46/p54) (n = 3); C, Dex activates p38 MAPK (n = 4). Cells were either left untreated or were treated with Dex (1 nM) for the indicated times. Levels of phospho-ERK, total ERK, phospho-JNK, total JNK, phospho-p38 MAPK, and total p38 MAPK protein were determined by immunoblotting as described in *Materials and Methods*. *Numbers above the images* represent the results (±SEM) of the densitometric analysis. *, $P \leq 0.05$ *vs.* untreated cells.

MAPK inhibitors were employed: PD98059 for MEK1, SP600125 for JNK, and SB203580 for p38 MAPK inhibition, respectively. ERK (Fig. 2A) and JNK (Fig. 2B) were found to mediate the induction of endothelial MKP-1 by Dex, but no involvement of p38 MAPK could be demonstrated (Fig. 2C). Because the PI3K/Akt pathway is known to mediate effects of glucocorticoids in endothelial cells (13, 14), we checked for an involvement of this pathway in the up-regulation of MKP-1. The PI3K inhibitor wortmannin was not able to inhibit the effect of Dex on MKP-1 protein expression (Fig. 2D), suggesting that the PI3K/Akt pathway does not play a crucial role.

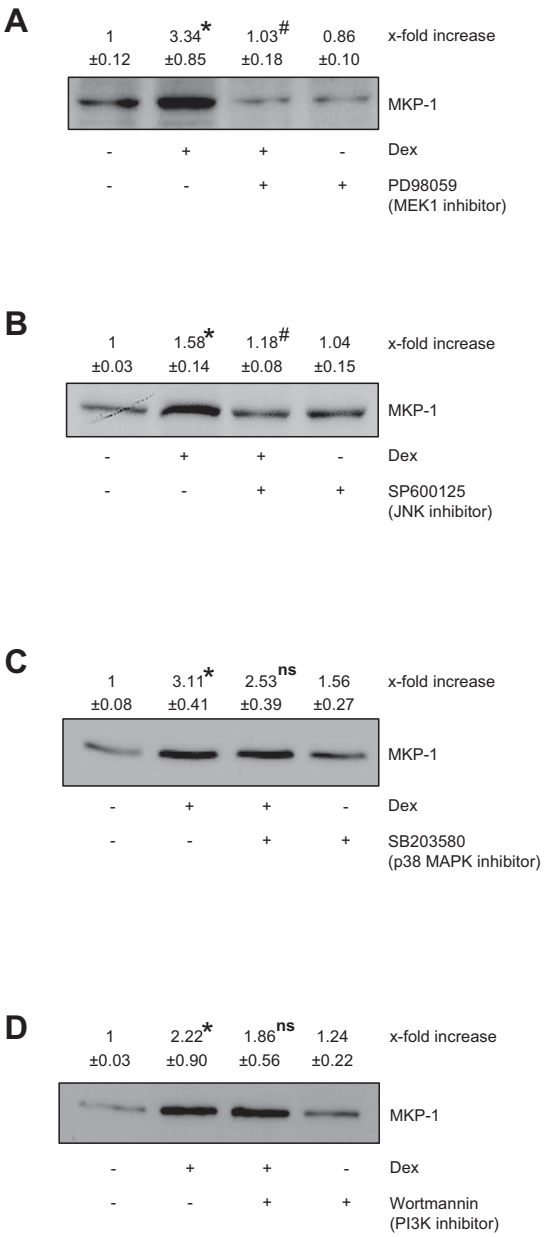


FIG. 2. ERK and JNK, but not p38 MAPK or PI3K, are crucially involved in the Dex-induced up-regulation of MKP-1. A, ERK is involved in the up-regulation of MKP-1 by Dex. Cells were either left untreated or treated with Dex (1 nM, 60 min) in the presence or absence of the MEK1 inhibitor PD98059 (10 $\mu$M, 60 min pretreatment) (n = 4). B, JNK is involved in the induction of MKP-1 by Dex. Cells were either left untreated or treated with Dex (1 nM, 60 min) in the presence or absence of the JNK inhibitor SP600125 (10 $\mu$M, 60 min pretreatment) (n = 4). C, p38 MAPK does not participate in Dex-induced increase of MKP-1 protein expression. Cells were either left untreated or treated with Dex (1 nM, 60 min) in the presence or absence of the p38 MAPK inhibitor SB203580 (10 $\mu$M, 60 min pretreatment) (n = 3). D, PI3K is not involved in the up-regulation of MKP-1 by Dex. Cells were either left untreated or treated with Dex (1 nM, 60 min) in the presence or absence of the PI3K inhibitor wortmannin (50 nM, 30 min pretreatment) (n = 3). Levels of MKP-1 protein were determined by immunoblotting as described in *Materials and Methods*. *Numbers above the images* represent the results (±SEM) of the densitometric analysis. *, $P \leq 0.05$ *vs.* untreated cells; #, $P \leq 0.05$ *vs.* cells treated with Dex only; ns, $P > 0.05$ *vs.* cells treated with Dex only.

### *ERK- and JNK-activated AP-1 plays a pivotal role in the up-regulation of MKP-1 by Dex*

An increase of MKP-1 protein levels is predominantly achieved by transcriptional mechanisms, *i.e.* the activation of transcription factors. Our hypothesis was that the transcription factor AP-1 could be involved. First, by performing EMSA, we checked whether Dex is able to activate AP-1. We found that AP-1 is rapidly (within 5 min) activated by Dex (Fig. 3A). This activation fades after 30 min, and a second activation peak emerges after 60 min, suggesting a biphasic Dex-induced AP-1 activity course. The role of AP-1 for the induction of MKP-1 was investigated via an AP-1 decoy approach: cells transfected with AP-1 decoy, but not with the scrambled decoy, lose the ability to elevate MKP-1 protein levels upon Dex treatment (Fig. 3B, *left panel*). The AP-1 decoys were proven to not influence basal protein levels of MKP-1 (Fig. 3B, *right panel*). These data point to a crucial role for AP-1 in the signaling pathway mediating the Dex-evoked induction of endothelial MKP-1. Moreover, we were interested in the question whether this Dex-induced AP-1 activity is caused by the Dex-induced activation of ERK or JNK. Using pharmacological inhibitors, both kinases were shown to mediate the Dex-induced AP-1 DNA-binding activity (Fig. 3C), suggesting that the signals of these two Dex-activated MAPKs converge on the AP-1 transcription factor.

### *ERK-activated CREB is also causally involved in the induction of MKP-1 by Dex*

We hypothesized that, besides AP-1, the transcription factor CREB participates in the induction of MKP-1. In fact, the CREB DNA-binding activity was increased by Dex within 5 min (Fig. 4A), and, comparable to the activation pattern of AP-1, the activity of CREB decreases after 15 min and a second activation peak appears within 60 min of Dex treatment. The causal link between the CREB activation and the induction of MKP-1 was established by using a CREB decoy approach; Dex is not able to increase MKP-1 protein levels in the presence of CREB decoy oligonucleotides (Fig. 4B, *left panel*). The CREB decoys were proven to not influence basal protein levels of MKP-1 (Fig. 4B, *right panel*). Because Dex activates ERK and ERK is known to influence CREB activity, we assumed that the Dex-induced ERK is involved in the activation of CREB. Indeed, we could show that the Dex-evoked increase of CREB DNA-binding activity is abrogated in endothelial cells treated with the MEK1 inhibitor PD98059 (Fig. 4C). These data suggest that the ERK-induced activation of CREB is pivotal for an up-regulation of MKP-1 by Dex.

### *The Dex-induced generation of ROS is crucial for the induction of MKP-1 and the activation of JNK*

We could recently demonstrate that ROS are able to up-regulate endothelial MKP-1 protein levels (12). Therefore, we hypothesized that Dex could induce MKP-1 via the formation of ROS. First, we tested whether Dex treatment leads to augmented ROS levels: Endothelial cells were loaded with an ROS-sensitive dye (dihydrofluorescein diacetate), and the

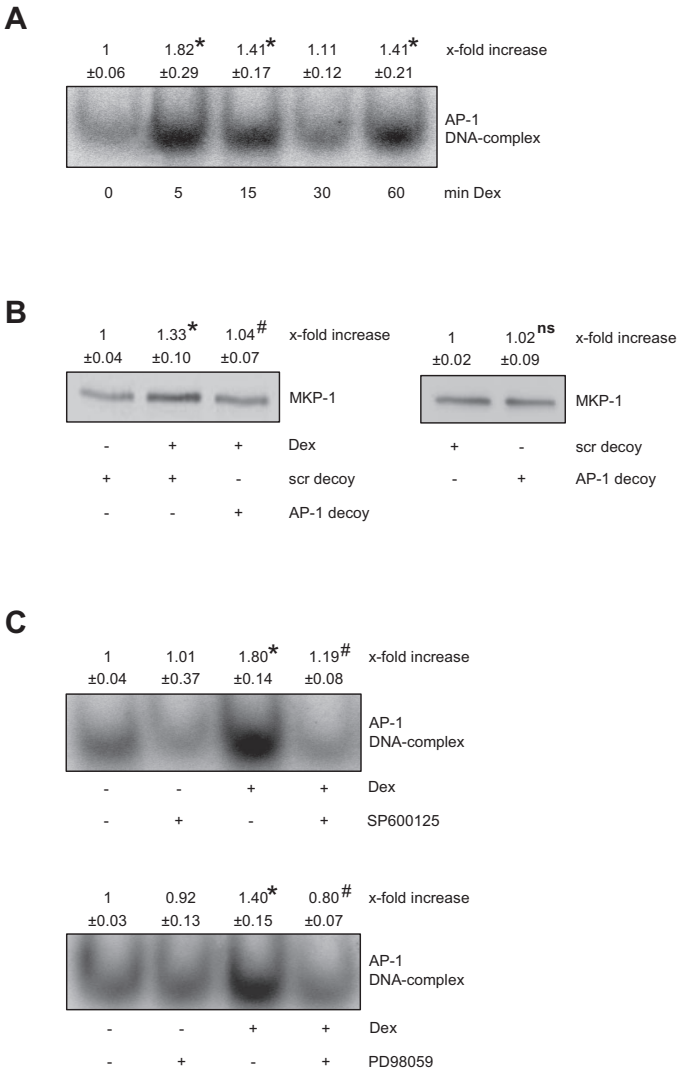


FIG. 3. ERK- and JNK-activated AP-1 plays a pivotal role in the induction of MKP-1 by Dex. A, Dex treatment leads to an activation of AP-1. Cells were either left untreated or were treated with Dex (1 nM) for the indicated times (n = 4). B, AP-1 is crucially involved in the up-regulation of MKP-1 by Dex. Cells were transfected with AP-1 decoy or scrambled decoy phosphorothioate oligonucleotides as described in *Materials and Methods*. *Left panel*, Cells were left untreated or treated with Dex (1 nM, 60 min) (n = 4); *right panel*, cells were left untreated to compare the influence of decoy treatment on basal MKP-1 protein levels (n = 4). C, ERK and JNK mediate the activation of AP-1 by Dex. Cells were either left untreated or treated with Dex (1 nM, 15 min) in the presence or absence of the MEK1 inhibitor PD98059 (10 $\mu$M, 60 min pretreatment) (n = 4) or the JNK inhibitor SP600125 (10 $\mu$M, 60 min pretreatment) (n = 3). AP-1 DNA-binding activity was determined by EMSA, and levels of MKP-1 protein were determined by immunoblotting as described in *Materials and Methods*. *Numbers above the images* represent the results (±SEM) of the densitometric analysis. *, $P \leq 0.05$ *vs.* untreated cells; #, $P \leq 0.05$ *vs.* cells treated with Dex only; ns, $P > 0.05$ *vs.* cells treated with scrambled decoy.

generation of ROS was measured upon treatment with different Dex concentrations (1–1000 nM). As shown in the *left panel* of Fig. 5A, Dex concentration-dependently increased the formation of endothelial ROS. Antioxidants, such as tiron and NAC, which effectively lower intracellular ROS levels

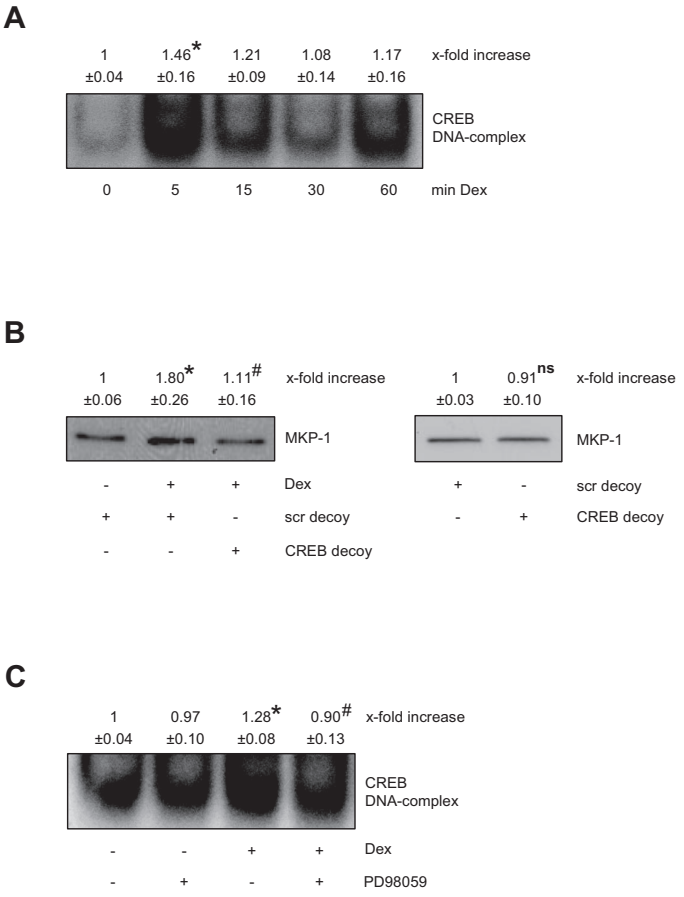


FIG. 4. ERK-activated CREB is also causally involved in the induction of MKP-1 by Dex. A, Dex treatment leads to an activation of CREB. Cells were either left untreated or treated with Dex (1 nM) for the indicated times (n = 4). B, CREB is crucially involved in the up-regulation of MKP-1 by Dex. Cells were transfected with CREB decoy or scrambled decoy phosphorothioate oligonucleotides as described in *Materials and Methods*. *Left panel*, Cells were left untreated or were treated with Dex (1 nM, 60 min) (n = 3); *right panel*, cells were left untreated to compare the influence of decoy treatment on basal MKP-1 protein levels (n = 4). C, ERK plays a pivotal role in the Dex-induced activation of CREB. Cells were either left untreated or treated with Dex (1 nM, 5 min) in the presence or absence of the MEK1 inhibitor PD98059 (10 $\mu$M, 60 min pretreatment) (n = 3). CREB DNA-binding activity was determined by EMSA, and levels of MKP-1 protein were determined by immunoblotting as described in *Materials and Methods*. *Numbers above the images* represent the results (±SEM) of the densitometric analysis. *, $P \leq 0.05$ *vs.* untreated cells; #, $P \leq 0.05$ *vs.* cells treated with Dex only; ns, $P > 0.05$ *vs.* cells treated with scrambled decoy.

(Fig. 5A, *right panel*), were able to prevent the Dex-induced increase of MKP-1 protein (Fig. 5B), pointing to a pivotal role for ROS in the up-regulation of MKP-1 by Dex. Moreover, because we found that ERK and JNK are responsible for the induction of MKP-1, we checked whether ROS are responsible for the activation of these kinases. In the presence of the antioxidant NAC, Dex forfeits its ability to activate JNK but not ERK (Fig. 5C), suggesting that the Dex-induced ROS formation is solely important for the activation of the JNK/AP-1 pathway, but not for the ERK/AP-1 and ERK/CREB pathway (Fig. 6).

## Discussion

In recent years, the phosphatase MKP-1 has increasingly been discussed as an interesting pharmacological target due to its role as a mediator of antiinflammatory properties (5–8). Moreover, MKP-1 has been shown to be a glucocorticoid-induced gene (3). Hence, the hypothesis of MKP-1 as a crucial transducer of the antiinflammatory effects of glucocorticoids has emerged and was corroborated by an *in vivo* study (9). In this context, we could recently show that MKP-1 represents a novel mediator of the antiinflammatory effects of glucocorticoids at low concentrations in human endothelial cells (4); Dex attenuates the TNF-$\alpha$-evoked E-selectin expression via an induction of MKP-1. Both MKP-1 mRNA and protein levels rise upon Dex treatment (maximal protein expression at 1 nM Dex after 60 min) (4). Despite this knowledge, the molecular mechanisms underlying the induction of MKP-1 are still very poorly investigated (3), and no data have as yet been available concerning the pathways by which Dex increases the expression of MKP-1 in the human endothelium. Thus, it is of great interest to clarify the signaling mechanisms Dex (1 nM, 60 min) uses to augment the cellular MKP-1 levels. We found that the transcription factors AP-1 and CREB are both necessary to increase the expression of MKP-1 upon Dex treatment. The activity of AP-1 is induced by the MAPKs ERK and JNK, and CREB is activated by JNK. For the activation of JNK, but not ERK, the Dex-evoked generation of ROS was found to play a crucial role (Fig. 6).

The finding that JNK and ERK are involved in the induction of MKP-1 is in accordance with studies that have proposed a negative feedback loop between MAPKs and MKP-1; *i.e.* activated MAPKs are able to induce MKP-1 to terminate their own activity (15–18). MAPKs play an intriguing role in the molecular pathways affected by glucocorticoids. On the one hand, MAPKs (particularly JNK and p38 MAPK) are well known to be strongly involved in the mediation of inflammatory effects of different stimuli (*e.g.* TNF-$\alpha$, IL-1, and lipopolysaccharide). In their role as inflammatory mediators, MAPKs are suppressed by glucocorticoids (19). On the other hand, MAPKs participate in the regulation of antiinflammatory actions, because our data show that they are involved in the induction of the antiinflammatory mediator MKP-1. In this role, MAPKs are activated by glucocorticoids. This ambiguous role highlights the fact that MAPKs are part of a highly complex spatiotemporal signaling network.

The regulation of MKP-1 activity is mainly achieved by influencing its transcription; MKP-1 is constitutively expressed at a low level and underlies, as an immediate-early gene product (20), a tight and rapid transcriptional up-regulation by different stimuli. In fact, Dex was shown to increase the expression of MKP-1 mRNA in our setting (4). We for the first time provide evidence that the transcription factors CREB (activated by ERK) and AP-1 (activated by JNK and ERK) are both necessary for the induction of endothelial MKP-1 by glucocorticoids. This is in line with a study demonstrating that the MKP-1 promoter region contains binding domains for both transcription factors (21). The fact that the blockade of each of these factors completely inhibits MKP-1 induction suggests a cooperative action of AP-1 and CREB. Our work for the first time describes the transcription factor

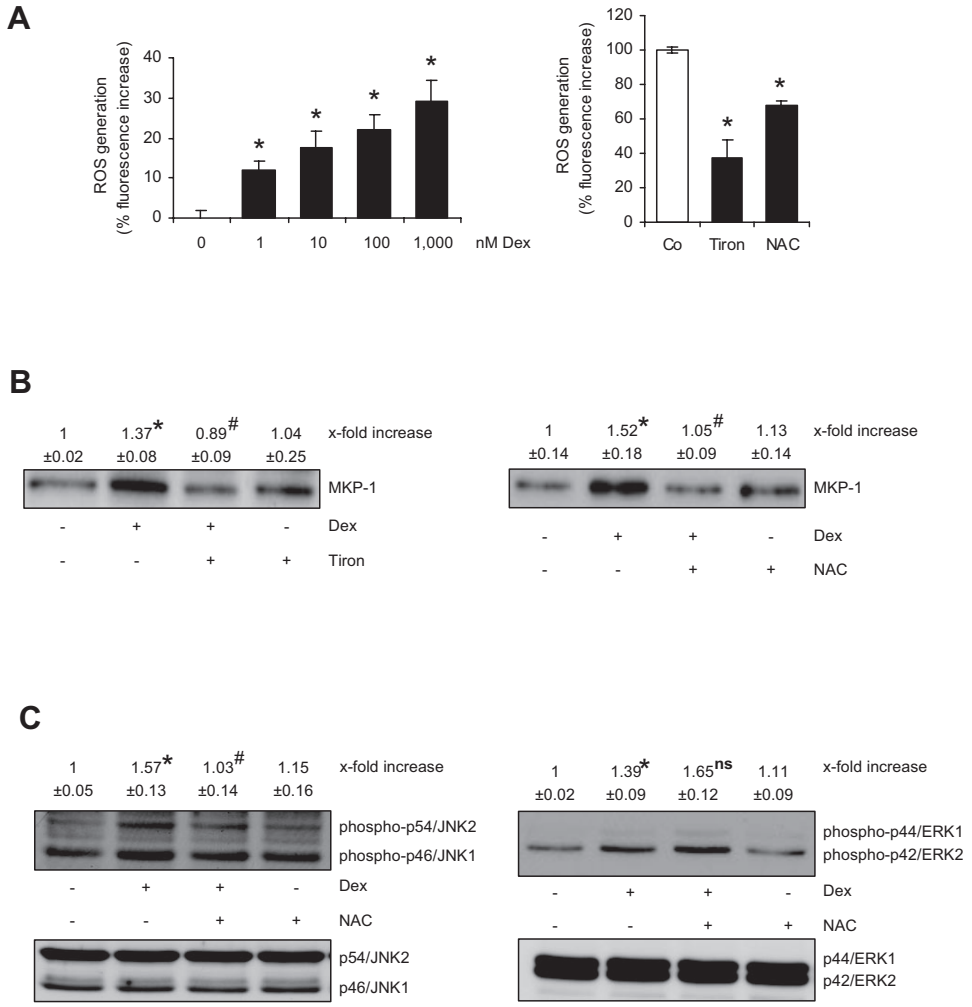


FIG. 5. Dex evokes formation of ROS, which is crucial for MKP-1 induction and JNK activation. A, *Left panel*, Dex induces ROS generation. Cells were either left untreated or treated with Dex (30 min) at the indicated concentrations (n = 2; eight replicates, each); *right panel*, antioxidants lower ROS generation. Cells were either left untreated or treated with tiron (10 mM, 30 min preincubation) or NAC (10 mM, 30 min preincubation) (n = 2). ROS generation was measured as described in *Materials and Methods*. B, Antioxidants inhibit the induction of MKP-1 by Dex. Cells were either left untreated or treated with Dex (1 nM, 60 min) in the presence or absence of the antioxidants tiron (10 mM, 30 min preincubation) (n = 6) or NAC (10 mM, 30 min preincubation) (n = 4). C, JNK, but not ERK, is activated by Dex-induced ROS. Cells were either left untreated or treated with Dex (1 nM, 15 min) in the presence or absence of the antioxidant NAC (10 mM, 30 min preincubation) (n = 4 for JNK; n = 4 for ERK). Levels of MKP-1, phospho-JNK, total JNK, phospho-ERK, and total ERK protein were determined by immunoblotting as described in *Materials and Methods*. *Numbers above the images* represent the results (±SEM) of the densitometric analysis. *, $P \leq 0.05$ *vs.* untreated cells; #, $P \leq 0.05$ *vs.* cells treated with Dex only; ns, $P > 0.05$ *vs.* cells treated with Dex only.

CREB as transducer of antiinflammatory actions of glucocorticoids in the vascular endothelium. This phenomenon has as yet been found only in lymphoblastic cells (22). AP-1 is known to be a typical proinflammatory transcription factor, which is inactivated by glucocorticoids via the classic mechanism of transrepression (1). Here, we present AP-1 as a mediator of antiinflammatory actions, because it induces MKP-1. This is in line with studies showing that AP-1 is able to transduce the antiinflammatory effects of natriuretic peptides (12) or salicylates (23). The controversial role of AP-1 could depend on the prevailing activation status of AP-1; Dex, natriuretic peptides, and salicylates were applied to resting, nonactivated endothelial cells, whereas the inhibitory role of glucocorticoids on AP-1 was investigated in inflammation-stressed cells.

Investigations into the mechanisms upstream of the MAPK activation revealed that Dex evokes the generation of ROS, which are crucially involved in the up-regulation of MKP-1 by activating JNK. It has to be stressed that the glucocorticoid-induced formation of ROS in endothelial cells has as yet been shown only in the context of side effects of a chronic glucocorticoid excess on the vascular system (24): The Dex-induced long-time ROS formation was regarded as a deleterious event leading to endothelial dysfunction. In contrast, we provide evidence that glucocorticoid-evoked ROS can also serve as important signaling molecules relaying antiinflammatory effects. This is in line with studies showing that ROS can be more than deleterious molecules, because they were also found to be important mediators or triggers of beneficial actions in endothelial cells (12, 25). Interestingly, the generation of ROS keeps rising with ascending Dex concentrations (1–1000 nM), whereas the induction of MKP-1 is maximal at 1 nM and dwindles at higher concentrations (>10 nM), as was previously reported by our group (4). In accordance with the above mentioned signaling functions of ROS, we speculate that only small amounts of ROS lead to an up-regulation of MKP-1 and that these small amounts could represent signaling ROS, which transduce an antiinflammatory signal. Higher ROS concentrations might participate in the action of Dex on different signaling pathways or might even be regarded as the above mentioned deleterious ROS that are associated with the side effects of a glucocorticoid therapy. This interesting issue needs further investigations to gain deeper insights into the detailed role of ROS in the signaling of Dex.

Glucocorticoid effects can be divided into long-term genomic effects (*i.e.* the translocation of the GR into the nucleus and the subsequent regulation of gene expression)

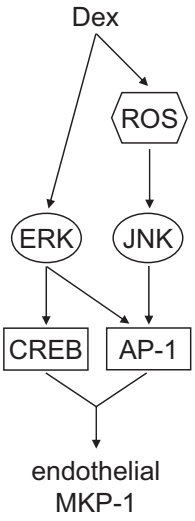


FIG. 6. Schematic overview of the signaling pathways by which Dex induces endothelial MKP-1.

and short-term nongenomic effects (*i.e.* glucocorticoids either nonspecifically alter the physicochemical properties of the cell membrane or specifically activate a cell membrane-associated or cytosolic GR) (26). By using a GR antagonist (RU486), we have previously demonstrated that the rapid Dex-induced increase of MKP-1 protein expression in human umbilical vein endothelial cells depends on the activation of the GR (4). Because the promoter region of MKP-1 does not contain glucocorticoid responsive element sequences (21), we assume that MKP-1 is up-regulated without an involvement of GR-DNA interaction but by an initiation of a rapid signaling cascade (ROS-MAPK-AP-1/CREB). Thus, we here describe a specific nongenomic effect of Dex in the human endothelium.

A growing number of studies report that nongenomic effects participate in the physiological or pharmacological action of steroid hormones. For glucocorticoids, effects such as the influence on intracellular calcium levels in rat pheochromocytoma cells (27), the inhibition of phagocytosis and superoxide production in mouse peritoneal macrophages (28), the blockage of human neutrophil degranulation (29), the focal adhesion kinase- and paxillin-regulated actions on the actin cytoskeleton in human endometrial adenocarcinoma cells (30), or the inhibition of the recruitment of signaling factors to activated epidermal growth factor receptors leading to a decreased liberation of arachidonic acid from human adenocarcinoma cells, were demonstrated to be nongenomic. Some of these effects have been suggested to depend on a membrane-associated GR. Interestingly, classic steroid receptors, such as the estrogen receptor (31), the progesterone receptor (32), or the mineralocorticoid receptor (33), have been found to signal at the plasma membrane in a nongenomic way. Because the nongenomic induction of MKP-1 completely depends on the classic GR, the question arises whether this GR is located in the cytosol or associated with the plasma membrane. In humans, membrane-bound GRs have as yet primarily been found in immune cells (34). The precise role of these receptors in mediating effects of glucocorticoids has still not been unraveled. Thus, clarifying whether a membrane-bound GR exists in endothelial cells and whether this receptor participates in the initiation of the studied signaling cascade is of great interest and warrants future investigations.

To the best of our knowledge, only two studies have as yet reported that glucocorticoids are able to induce a nongenomic, GR-dependent effect in endothelial cells. High-dose Dex was found to exert cardiovascular protection [*i.e.* decreased vascular inflammation and reduced myocardial infarct size after ischemia/reperfusion injury (13)] and neuroprotective effects [*i.e.* augmented cerebral flow and reduced cerebral infarct size after transient cerebral ischemia (14)]. Both phenomena are based on the activation of endothelial NO synthase and involve a nongenomic but GR-dependent activation of the PI3K/Akt pathway (13, 14). Thus, we hypothesized that this pathway could also participate in the induction of MKP-1. However, no involvement of PI3K in the Dex-induced up-regulation of MKP-1 could be detected.

The genomic effects of glucocorticoids are generally thought to be the main cause for side effects limiting their therapeutic use. To gain novel therapeutic options, *i.e.* to find new antiinflammatory principles and, thus, pharmacological targets, it is of great importance to investigate alternative pathways by which the antiinflammatory properties of glucocorticoids are mediated. Therefore, the basic research dealing with nongenomic effects has recently been brought into the focus (34). In this regard, our data add new and valuable information to this field, because we elucidated the molecular mechanisms by which MKP-1, a novel antiinflammatory mediator used by glucocorticoids, is induced in the human vascular endothelium.

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