

**NF- $\kappa$ B-independent Anti-inflammatory Action of Salicylate in Human Endothelial Cells:**

**Induction of Heme Oxygenase-1 by the JNK/AP-1 Pathway**

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Abbreviations:

HUVEC, human umbilical vein endothelial cells; IL-4, interleukin-4; NaSal, sodium salicylate; HO-1,

heme oxygenase-1; NF- $\kappa$ B, nuclear factor-kappa B; JNK, c-jun N-terminal kinase;

AP-1, activator protein-1; COX, cyclooxygenase; MAPK, mitogen-activated protein kinase; NFAT,

nuclear factor of activated T cells;

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## ABSTRACT

In contrast to aspirin, salicylate, its active metabolite, possesses profound anti-inflammatory properties without blocking cyclooxygenase. Inhibition of the transcription factor NF- $\kappa$ B has been discussed to play a role in the anti-inflammatory profile of salicylate. However, in addition, NF- $\kappa$ B-independent effects of salicylate have been assumed, but have as yet been poorly investigated. Therefore, aim of the present study was to investigate NF- $\kappa$ B-independent anti-inflammatory mechanisms of salicylate in human umbilical vein endothelial cells (HUVEC) using IL-4 as NF- $\kappa$ B-independent pro-inflammatory stimulus and P-selectin as inflammatory read-out parameter. Using quantitative real-time RT-PCR, we found that salicylate decreases IL-4-induced P-selectin expression. As judged by Western blot analysis, salicylate increased endothelial heme oxygenase-1 (HO-1) protein levels. Employing both the HO-1 inhibitor SnPP and HO-1 antisense oligonucleotides, we causally linked the induction of HO-1 to the decrease of P-selectin. Moreover, we were interested in the signaling mechanisms leading to the upregulation of HO-1 by salicylate. JNK was found to be activated by salicylate and we could causally link this activation to the induction of HO-1 by using the JNK inhibitor SP600125. By applying AP-1 decoys, it was shown that the transcription factor AP-1 is crucially involved in the upregulation of HO-1 downstream of JNK. In summary, our study introduces HO-1 as novel NF- $\kappa$ B-independent anti-inflammatory target of salicylate in human endothelial cells. Moreover, we elucidated the JNK/AP-1 pathway as crucial for the induction of HO-1 by salicylate.

## INTRODUCTION

Aspirin – acetyl salicylic acid – is the most widely used drug in the world, since it possesses profound analgesic, anti-thrombotic, and anti-inflammatory properties (Vane and Botting, 2003). For many years, these properties have been ascribed to the ability of aspirin to block prostaglandin synthesis by inhibition of cyclooxygenase (COX) (Vane and Botting, 2003). However, several lines of evidence point to COX-independent anti-inflammatory effects: The doses of aspirin needed to medicate chronic inflammatory disorders are much higher than those required to inhibit prostaglandin synthesis. Salicylate – the active metabolite of aspirin – rather than the rapidly deacetylated aspirin is considered to exert anti-inflammatory effects and does not inhibit COX (Tegeger, et al., 2001). In fact, salicylate is known to impair the activation of the transcription factor NF- $\kappa$ B (Kopp and Ghosh, 1994), which plays a crucial role in the regulation of many pro-inflammatory genes. Nevertheless, the inhibition of NF- $\kappa$ B was not proven to be of importance for the anti-inflammatory effects of salicylate *in vivo* (Cronstein, et al., 1999). Therefore, NF- $\kappa$ B-independent effects of salicylate have been assumed, but have as yet been poorly investigated.

The endothelium is an important regulator of inflammatory events, since it plays a crucial role in the attraction, adhesion, and migration of leukocytes to sites of inflammation (Biedermann, 2001). Due to this important role for endothelial cells in inflammatory events, we aimed to investigate NF- $\kappa$ B-independent effects of salicylate in human endothelial cells. We chose interleukin-4 (IL-4) as pro-inflammatory stimulus. IL-4 is an immunomodulatory cytokine secreted by activated T cells, eosinophils, and mast cells and it plays an important role in the pathology of chronic inflammatory disorders, such as asthma (Bradding, et al., 1992) or atherosclerosis (Sasaguri, et al., 1998). Moreover, IL-4 is known to induce genes that are generally associated with an activated, pro-inflammatory status of the endothelium, such as P-selectin (Yao, et al., 1996). We chose this molecule as inflammatory read-out parameter in order to analyze the effects of salicylate on the IL-4-induced, NF- $\kappa$ B-independent activation of human endothelial cells.

Heme oxygenase-1 (HO-1) is the rate-limiting enzyme in heme degradation. It catalyzes the cleavage of heme to yield free iron, carbon monoxide, and biliverdin. HO-1 has increasingly been recognized to possess a wide range of biological effects, since it is tightly involved in both physiological as well as pathophysiological processes, such as atherogenesis (Ishikawa and Maruyama, 2001) and ischemia-reperfusion injury (Tsuchihashi, et al., 2004). In the recent past, there has been accumulating evidence that HO-1 possesses profound anti-inflammatory features (Alcaraz, et al., 2003). Since the transcription factor AP-1 has been shown by ourselves to induce HO-1 expression in endothelial cells and

Thus, aim of the present study was to investigate an NF- $\kappa$ B-independent anti-inflammatory mechanism of salicylate in primary human umbilical vein endothelial cells (HUVEC) using IL-4 as pro-inflammatory stimulus and P-selectin as inflammatory read-out parameter. We hypothesized that salicylate is able to induce HO-1 and that this induction is able to attenuate the IL-4-evoked increase of P-selectin. Moreover, we sought to clarify the underlying signaling pathways by which HO-1 is induced by salicylate.

## METHODS

**Materials.** Human recombinant IL-4 and TNF- $\alpha$  were from Sigma-Aldrich (Taufkirchen, Germany). The HO-1 inhibitor tin(II) protoporphyrin IX (SnPP) (for reference see (Drummond and Kappas, 1981)) was from Alexis (Grünberg, Germany) and sodium salicylate (NaSal) was from Fluka (Buchs, Switzerland). The JNK inhibitor 1,9-pyrazoloanthrone (SP600125) (for reference see (Bennett, et al., 2001)) was from Calbiochem (Schwalbach, Germany). Mouse monoclonal anti-human HO-1 antibody (clone 23) was from BD Biosciences (Heidelberg, Germany). Mouse monoclonal anti-human phospho-JNK (Thr183/Tyr185) antibody (clone G9) and horseradish peroxidase-conjugated goat anti-mouse antibody were from Cell Signaling/New England Biolabs (Frankfurt/Main, Germany). Primers for P-selectin and GAPDH were from Invitrogen (Karlsruhe, Germany). TaqMan probes for P-selectin and GAPDH were from Applied Biosystems (Hamburg, Germany).

**Cell Culture.** Human umbilical vein endothelial cells (HUVEC) were prepared by digestion of umbilical veins as previously described (Kiemer, et al., 2002a). Cells were cultured in Endothelial Cell Growth Medium (Promocell, Heidelberg, Germany) supplemented with 10% heat-inactivated fetal calf serum (Biochrom, Berlin, Germany) and used for all experiments at passage 3. Cells were routinely tested for mycoplasma contamination with the PCR detection kit VenorGeM (Minerva Biolabs, Berlin, Germany).

**Measurement of P-selectin mRNA levels by quantitative real time reverse transcription-polymerase chain reaction (RT-PCR).** Cells were grown in 6-well plates until confluence and treated as indicated in the respective figure legend. Extraction of total mRNA was performed with the RNeasy Mini Kit (Qiagen, Hilden, Germany). RNase-free DNase was applied for DNase digestion (RNase-free DNase Set, Qiagen). Reverse transcription was carried out using the RNA PCR Core Reagent Kit (Applied Biosystems, Hamburg, Germany) in a GeneAmp PCR system 9700 (Applied Biosystems). Real time PCR was performed using the TaqMan PCR Core Reagent Kit (Applied Biosystems) in a GeneAmp 5700 Sequence

Detection System (Applied Biosystems). P-selectin forward primer: 5'-TGAAGGAAGGTTTTCTCCAC-TTTG-3'; reverse primer: 5'-AGACTCCAGAAGATGCTACAGGAATT-3'; probe: 5'-TGGAAAGCAGGTGGCATCTCTAATTGGA-3'. GAPDH forward primer: 5'-GGGAAGGTGAAGGTCGGAGT-3'; reverse primer: 5'-TCCACTTTACCAGAGTTAAAAGCAG-3'; probe: 5'-ACCAGGCGCCCAATACG-ACCAA-3'. Results were quantified based on the relative expression of the P-selectin gene vs. the housekeeping gene GAPDH using the mathematical model for relative quantification according to Pfaffl (Pfaffl, 2001).

**Measurement of HO-1 and phospho-JNK protein expression by Western blot analysis.** Cells were grown in 6-well plates until confluence and were treated as indicated in the respective figure legend. Western blot analysis was performed as described (Kierner, et al., 2002a). HO-1 antibody and phospho-JNK antibody (see "Materials") were diluted 1:250 and 1:1000, respectively. For densitometric analysis the Kodak 1D software version 3.5.4 was used.

**Measurement of NF- $\kappa$ B and AP-1 DNA-binding activity by electrophoretic mobility shift assay (EMSA).** Cells were grown in 6-well plates until confluence and were treated as indicated in the respective figure legend. Nuclear extracts were prepared and EMSA was performed as described (Kierner, et al., 2002b). Consensus binding-sequence for AP-1: 5'-CGCTTGATGAGTCAGCCGGAA-3' (Promega, Mannheim, Germany) and for NF- $\kappa$ B: 5'-AGTTGAGGGGACTTCCCAGGC-3' (Promega). For densitometric analysis the OptiQuant software version 4.00 was used.

**HO-1 antisense and AP-1 decoy experiments.** Cells were grown in 6-well plates until 80% confluence and were transfected with HO-1 antisense phosphorothioate oligonucleotides (antisense: 5'-CGCCTTCATGGTGCC-3'; sense: 5'-GGCACCATGAAGGCG-3') according to (Wagener, et al., 1999) or AP-1 decoy phosphorothioate oligonucleotides (decoy: 5'-CGCTTGATGACTCAGCCGGAA-3'; scrambled decoy: 5'-CGCTTGATGACTTGGCCGGAA-3') according to (Jan, et al., 2000) by using the

jetPEI-RGD transfection reagent (Polyplus-Transfections/Biomol, Hamburg, Germany). Cells were treated for 4 h with the DNA-jetPEI-RGD-complexes. Experiments were performed 24 h (HO-1 antisense) or 3 h (AP-1 decoy) after transfection.

**Statistical analysis.** Unless stated otherwise all experiments were done from at least three different cell preparations in at least duplicates. Data are expressed as mean  $\pm$  SEM. Statistical analysis was performed with the GraphPad Prism software version 3.03. To compare three or more groups, one-way ANOVA followed by Neuman-Keuls' post hoc test was used.



## RESULTS

**IL-4 does not influence NF- $\kappa$ B DNA-binding activity.** To confirm the lack of NF- $\kappa$ B activation for our experimental setting, we treated HUVEC with IL-4 and analyzed NF- $\kappa$ B DNA-binding activity. TNF- $\alpha$  – a well-known activator of NF- $\kappa$ B – served as a positive control: TNF- $\alpha$  dramatically increased NF- $\kappa$ B activity (Fig. 1A). However, IL-4 did not alter NF- $\kappa$ B DNA-binding activity at any time point (Fig. 1A). This finding confirms that IL-4 does not activate NF- $\kappa$ B in human endothelial cells.

**The IL-4-evoked increase of P-selectin is reduced by NaSal.** P-selectin – a product of a gene involved in endothelial inflammation – is known to be induced by IL-4 in HUVEC. We confirmed this in our experimental setting (Fig. 1B). Pre-treatment with sodium salicylate (NaSal, 10 mM) strongly reduced the expression of P-selectin (Fig. 1B).

**NaSal induces endothelial HO-1.** Since we hypothesized that HO-1 could be responsible for the observed NF- $\kappa$ B-independent actions of NaSal, we first investigated whether NaSal is able to induce HO-1 protein expression in primary human endothelial cells. In fact, treatment with NaSal led to an increase of HO-1 protein expression with maximal protein levels at about 60 min (Fig. 2A) and 10 mM (Fig. 2B).

**HO-1 is involved in the decrease of IL-4-induced P-selectin by NaSal.** Since we observed an upregulation of HO-1 by NaSal, we assumed that HO-1 might be involved in the decrease of IL-4-induced P-selectin expression by NaSal. We treated HUVEC with the HO-1 blocker SnPP and found that the NaSal-evoked inhibition of P-selectin expression was clearly diminished (~50% reversal) (Fig. 3A). Moreover, we performed HO-1 antisense experiments and revealed that an attenuated induction of HO-1 protein expression due to the presence of antisense oligonucleotides leads to a diminished reduction of P-selectin levels (~30% reversal) (Fig. 3B). Control experiments confirmed that the used HO-1 antisense

oligonucleotides are in fact able to attenuate endothelial HO-1 protein levels (Fig. 3C). Our findings suggest an involvement of NaSal-induced HO-1 in the decrease of IL-4-evoked P-selectin expression.

**NaSal induces HO-1 protein expression via JNK.** We aimed to elucidate the underlying signaling mechanism by which salicylate leads to an upregulation of endothelial HO-1 protein levels. We hypothesized that MAPK could be involved. Indeed, as shown by the detection of the phosphorylated form of the MAPK JNK (phospho-p54/JNK2 and phospho-p46/JNK1), NaSal activated JNK after 15 min. The amount of phosphorylated JNK returned to almost basal levels after 60 min (Fig. 4A). Activation of JNK was found to be crucial for the upregulation of HO-1, since the pharmacological JNK inhibitor SP600125 abrogated the NaSal-induced increase of HO-1 protein levels (Fig. 4B). These findings point to an involvement of JNK in the mechanism by which NaSal induces HO-1.

**NaSal induces HO-1 protein expression via AP-1.** Downstream of JNK, we wanted to clarify whether the transcription factor AP-1 might be involved in the induction of HO-1 by NaSal. In fact, treatment of HUVEC with NaSal led to strong time-dependent activation of AP-1 DNA-binding activity (Fig. 5A). Furthermore, transfection of HUVEC with AP-1 decoys completely blocked the ability of NaSal to induce HO-1 (Fig. 5B), suggesting that AP-1 activation is crucial for the salicylate-induced increase of HO-1 protein expression.

## DISCUSSION

Salicylate possesses profound anti-inflammatory effects, but it does not inhibit cyclooxygenase (COX) (Tegeeder, et al., 2001). Therefore, COX-independent pathways have been postulated. Most attention has been paid to a salicylate-evoked inhibition of the pro-inflammatory transcription factor NF- $\kappa$ B (Kopp and Ghosh, 1994), although an influence of salicylate on other signal transduction systems commonly associated with inflammatory events, like the MAPK pathway (Pillinger, et al., 1998), the AP-1 pathway (Dong, et al., 1997), and the NFAT pathway (Aceves, et al., 2004) have also been shown to contribute to the anti-inflammatory profile of salicylate. In the present work we provide for the first time evidence that heme oxygenase-1 (HO-1) is a further NF- $\kappa$ B-independent player in the complex concert of anti-inflammatory mechanisms evoked by salicylate.

For the following reasons we applied sodium salicylate at high concentrations (10 mM): according to (Amann and Peskar, 2002), anti-inflammatory therapy with aspirin (which rapidly deacetylates to its active metabolite salicylate) results in plasma salicylate concentrations of about 2 mM. Moreover, salicylate is known to accumulate in inflamed tissue (Brune, 1977). Unfortunately, no data exists about the precise concentrations of salicylates in inflamed tissue, but it seems obvious that high concentrations of salicylate – as employed in our *in vitro* model – are required to mimic the *in vivo* situation of an effective anti-inflammatory action. To investigate potential cytotoxic effects evoked by 10 mM salicylate, we performed CellTiter Blue cell viability assays and thus, excluded remarkable cytotoxicity at this concentration (data not shown).

Aiming to identify NF- $\kappa$ B-independent mechanisms, we used interleukin-4 (IL-4) as pro-inflammatory stimulus and confirmed that no influence on NF- $\kappa$ B was exerted by IL-4. This is in accordance with data from other groups showing no alteration of NF- $\kappa$ B activity upon treatment of endothelial cells with IL-4 (Xia, et al., 1998; McCarty, et al., 1995). Importantly, salicylate was found to block the IL-4-induced increase of the used read-out parameter for endothelial inflammation, P-selectin. Inhibition of P-selectin

by salicylate has previously been observed (Xia, et al., 1998) and was confirmed in our experimental setting.

In terms of the salicylate-evoked inhibition of P-selectin expression, we show for the first time that salicylate is able to induce HO-1 protein expression in primary human endothelial cells and that the observed HO-1 induction is a feature linked to the inhibition of P-selectin by salicylate. However, it should be kept in mind that neither the HO-1 inhibitor nor the HO-1 antisense approach did completely reverse the effect of salicylate. Although additional NF- $\kappa$ B-independent anti-inflammatory pathways targeted by salicylate have to be discussed in the context of our data, our study introduces HO-1 as a novel NF- $\kappa$ B-independent molecular target of salicylate in endothelial inflammatory processes. This finding is in line with many studies that link HO-1 to anti-inflammatory features (Wagener, et al., 2003) and is strengthened by observations made in the case of HO-1-deficiency (Yachie, et al., 1999). Two recently published studies (Nascimento-Silva, et al., 2005; Grosser, et al., 2003) showing the ability of aspirin to induce HO-1 in ECV304 also support our data.

Based on the novel role for salicylate-induced HO-1 in the endothelium, the underlying signaling mechanisms were of interest. We could show that salicylate increases HO-1 protein expression in human endothelial cells via activation of JNK and the transcription factor AP-1. Experiments employing a pharmacological JNK inhibitor (SP600125) (Bennett, et al., 2001) and inhibiting DNA-binding of AP-1 by decoy oligonucleotides (Jan, et al., 2000) in fact indicate a crucial role for the JNK/AP-1 pathway in the signaling events responsible for the upregulation of HO-1. In support of our findings, JNK activation by salicylate has also been observed in human eosinophils (Wong, et al., 2000), HO-29 colon cancer (Schwenger, et al., 1999), and COS-1 cells (Schwenger, et al., 1999). Interestingly, salicylate inhibits an already activated JNK in fibroblasts and mouse epidermal cells (Schwenger, et al., 1997; Huang, et al., 1997), suggesting that the effects of salicylate on JNK activity highly depend both on the cellular context and on the activity status of JNK in the current experimental setting. Our results are also supported by the finding that the human HO-1 gene contains AP-1 binding sites (Lavrovsky, et al., 1994) and by corresponding data for some other stimuli of HO-1 induction (Kierner, et al., 2003; Terry, et al., 1998).

However, our finding that salicylate induces DNA-binding of AP-1 differs from most of the published data, which describe an inhibitory effect of salicylates on AP-1. In mouse epidermal cells, aspirin and salicylate have been shown to inhibit epidermal growth factor-induced AP-1 activation and also basal AP-1 levels (Dong, et al., 1997). Also in human cell systems salicylates have shown inhibitory action on AP-1, for instance in human cervical cancer cells (Muroso, et al., 2000). All this seems to be in contrast to our findings. However, all these opposing studies show inhibitory effects of salicylates only in settings where the cells have already been activated by diverse stimuli. Publications reporting basal stimulatory effects on the JNK/AP-1 pathway are rare (Vartiainen, et al., 2003; Wong, et al., 2000; Schwenger, et al., 1999) and none is available for the human endothelium. Thus, to the best of our knowledge, this is the first report proving that activation of the JNK/AP-1 signaling pathway is crucial for the salicylate-induced HO-1 expression in the human endothelium.

Due to this involvement of the JNK/AP-1 pathway, the question raises how salicylate is able to activate JNK. Interestingly, salicylate has recently been described to evoke oxidative stress (Battaglia, et al., 2005), which could account for the activation of JNK that represents a classical stress-activated protein kinase (Adler, et al., 1999). To test this hypothesis, we measured the generation of reactive oxygen species (ROS) as previously described (Fürst, et al., 2005). However, we could not detect an increase in ROS formation upon salicylate treatment (data not shown). We can only speculate that salicylate might activate a kinase upstream of JNK or inhibit a MAPK phosphatase.

In summary, the present study identifies HO-1 as an NF- $\kappa$ B-independent mediator in the anti-inflammatory signaling concert triggered by salicylate in primary human endothelial cells. Thereby, the JNK/AP-1 pathway is crucially involved in the upregulation of endothelial HO-1. The data add further evidence to the fact that salicylate causes anti-inflammatory effects independent of COX activity and, moreover, not related to NF- $\kappa$ B inhibition.

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## LEGENDS FOR FIGURES

### Figure 1

A, IL-4 does not influence NF- $\kappa$ B DNA-binding activity. Cells were either left untreated (Co) or were treated with IL-4 (10 ng/ml) for the indicated times. Treatment with TNF- $\alpha$  (10 ng/ml, 30 min) served as positive control. NF- $\kappa$ B DNA-binding activity was analyzed by electrophoretic mobility shift assay (EMSA) as described in the section “Materials and Methods”. B, The induction of P-selectin by IL-4 is reduced by NaSal. Cells were either left untreated (Co) or were treated with IL-4 (10 ng/ml) for 20 h with or without pre-treatment with NaSal (10 mM) for 60 min. Levels of P-selectin mRNA were determined by quantitative real-time RT-PCR as described in the section “Materials and Methods”. \* $p < 0.001$  compared with Co. # $p < 0.001$  compared with IL-4.

### Figure 2

Induction of HO-1 protein expression by NaSal. A, Cells were either left untreated (Co) or were treated with NaSal (10 mM) for the indicated times. B, Cells were either left untreated (Co) or were treated with the indicated concentration of NaSal for 60 min. HO-1 protein levels were determined by Western blot analysis as described in the section “Materials and Methods”. \* $p < 0.01$  compared with Co.

### Figure 3

A, The HO-1 inhibitor SnPP reduces the NaSal-evoked decrease of IL-4-induced P-selectin expression. Cells were either left untreated or were treated with IL-4 (10 ng/ml) for 20 h with or without pre-treatment with NaSal (10 mM) for 60 min. SnPP (10  $\mu$ M) was applied 30 min before NaSal. Levels of P-selectin mRNA were determined by quantitative real time RT-PCR as described in the section “Materials and Methods”. B, Silencing of HO-1 reduces the NaSal-evoked decrease of IL-4-induced P-selectin expression. Cells were either left untreated or were treated with NaSal (10 mM) for 60 min in the

presence of HO-1 antisense or sense oligonucleotides. Levels of P-selectin mRNA were determined by quantitative real time RT-PCR as described in the section “Materials and Methods”. C, Functionality of the HO-1 antisense approach. Cells were either left untreated or were treated with NaSal (10 mM) for 60 min in the presence of HO-1 antisense or sense oligonucleotides. HO-1 protein levels were determined by Western blot analysis as described in the section “Materials and Methods”. Antisense experiments were performed as described in the section “Materials and Methods”. \* $p < 0.001$  compared with the IL-4/IL-4 + HO-1 sense group. # $p < 0.001$  compared with the IL-4 + NaSal + SnPP/HO-1 antisense group. + $p < 0.05$  compared with the only HO-1 sense-treated group.

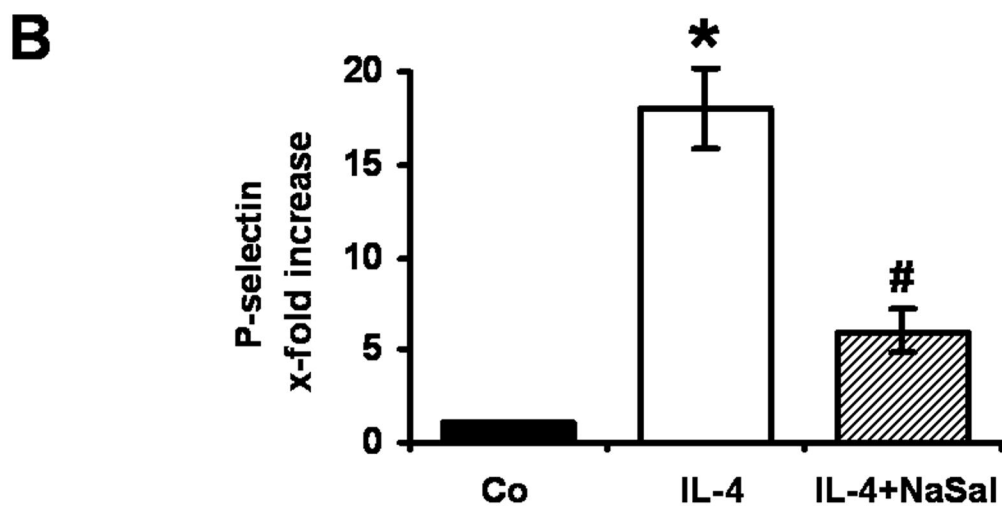
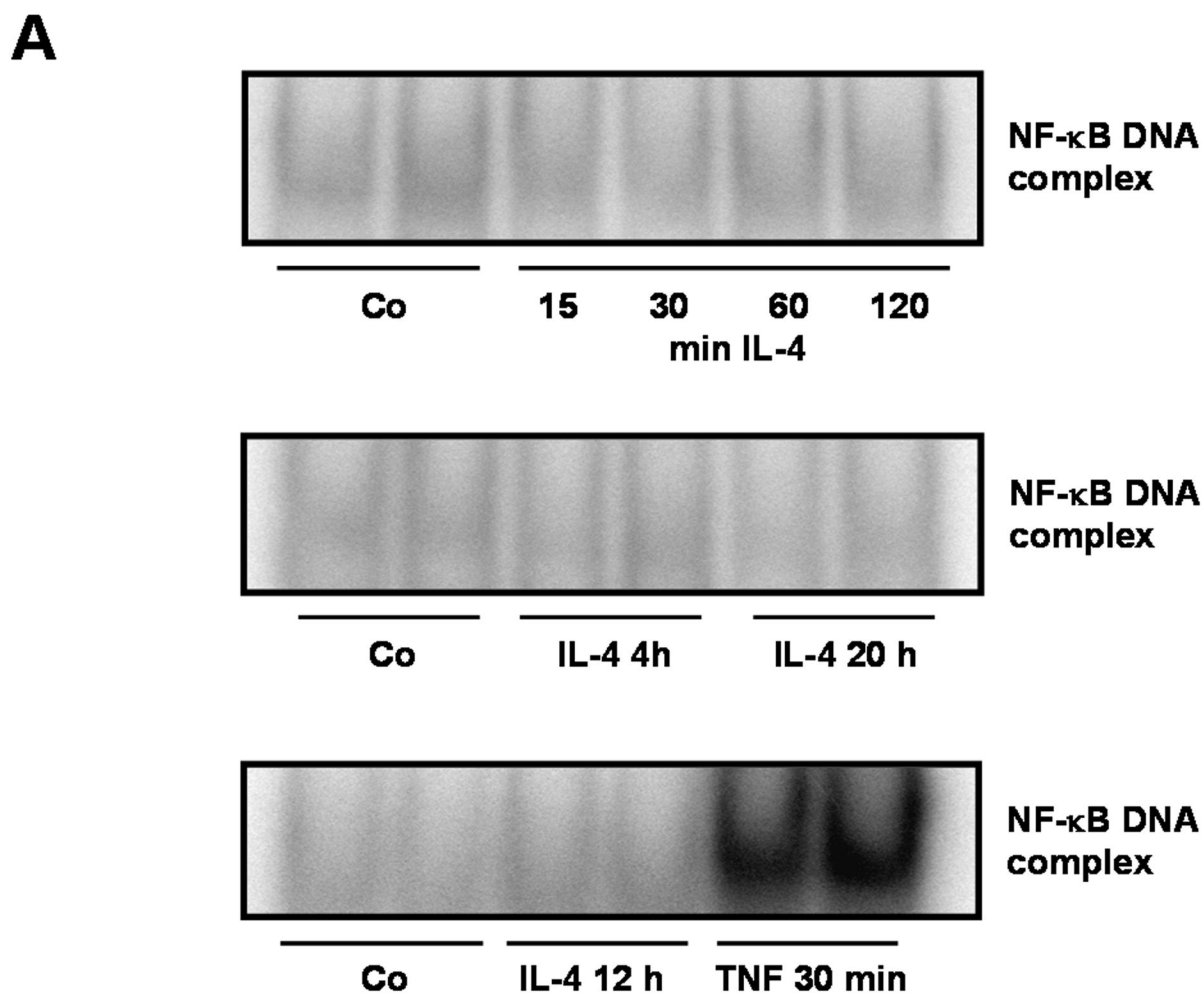
#### Figure 4

A, NaSal activates JNK. Cells were either left untreated (Co) or were treated with NaSal (10 mM) for the indicated times. TNF- $\alpha$  (10 ng/ml, 30 min) served as positive control. B, JNK is crucially involved in the induction of HO-1 by NaSal. Cells were either left untreated or were treated with NaSal (10 mM) for 60 min. The JNK inhibitor SP600125 (SP, 10  $\mu$ M) was added 60 min before NaSal. Phospho-JNK and HO-1 protein levels were determined by Western blot analysis as described in the section “Materials and Methods”. \* $p < 0.01$  compared with the untreated group. # $p < 0.001$  compared with NaSal.

#### Figure 5

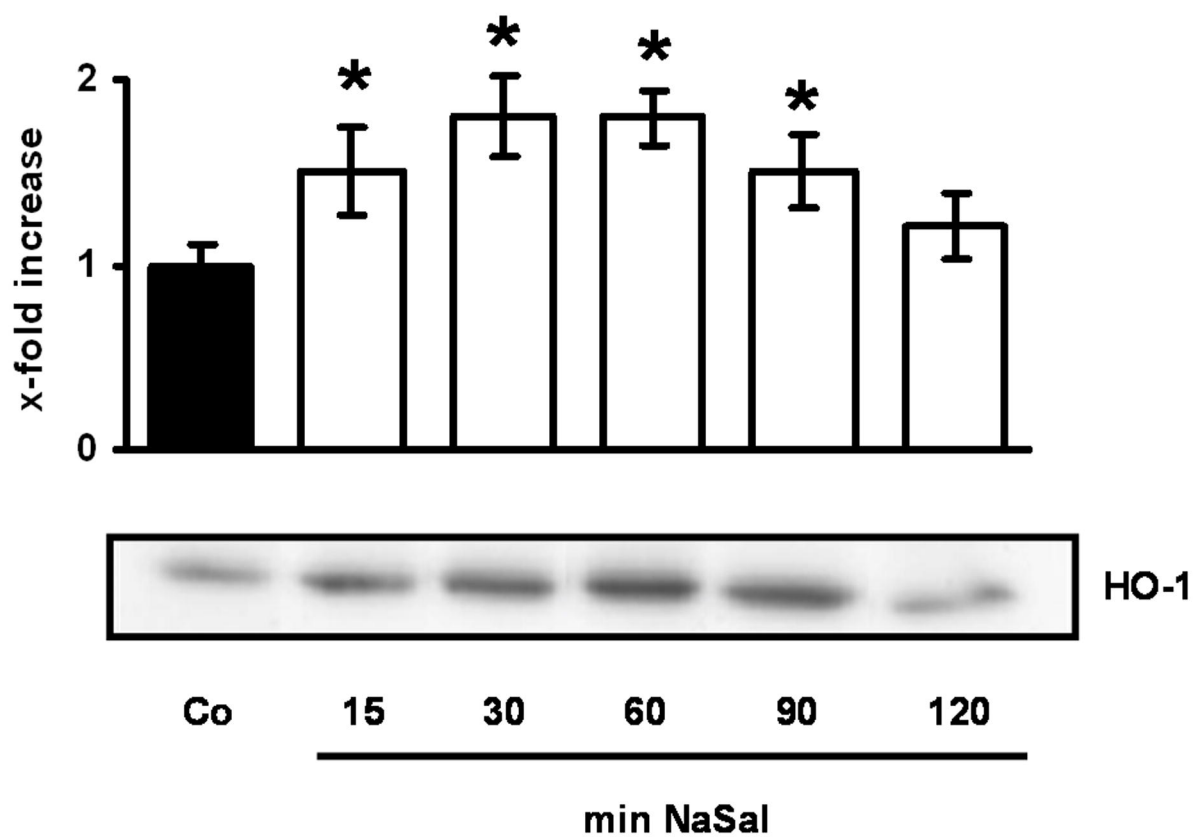
A, NaSal activates AP-1. Cells were either left untreated (Co) or were treated with NaSal (10 mM) for the indicated times. AP-1 DNA-binding activity was assessed by EMSA as described in the section “Materials and Methods”. B, AP-1 is crucially involved in the induction of HO-1 by NaSal. Cells were either left untreated or were treated with NaSal (10 mM) for 60 min in the presence of AP-1 decoys or scrambled (scr) decoys. Decoy experiments were performed as described in the section “Materials and Methods”. HO-1 protein levels were determined by Western blot analysis as described in the section “Materials and Methods”. \* $p < 0.01$  compared with Co. # $p < 0.001$  compared with the NaSal + scr AP-1 decoy group.

# Figure 1

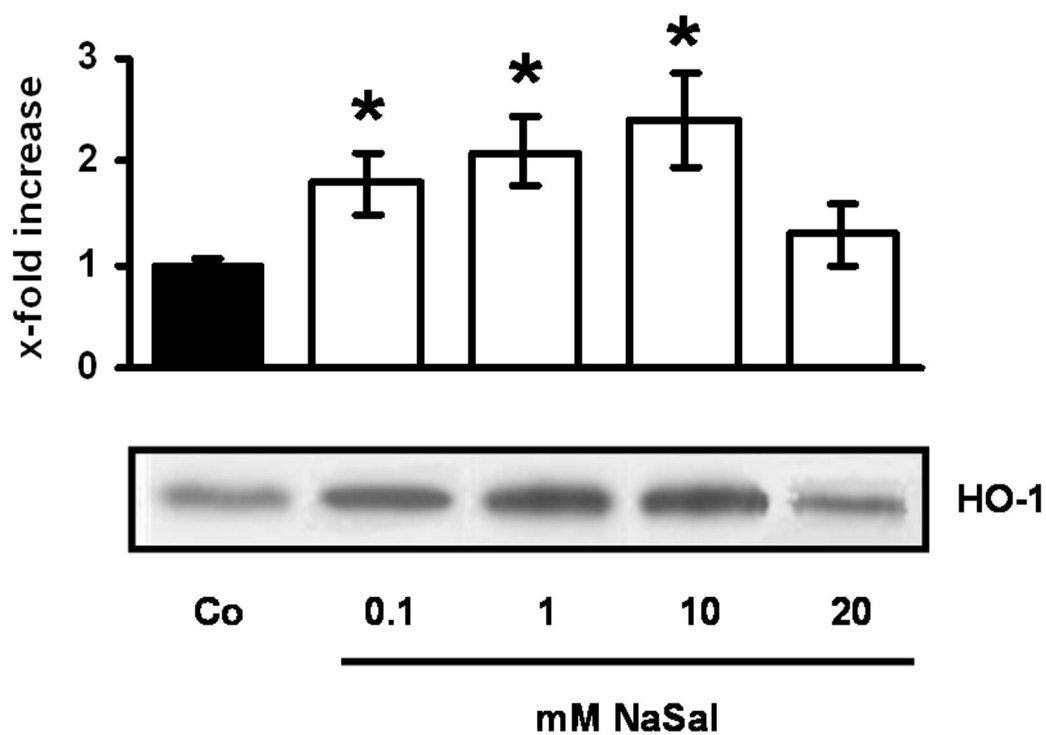


# Figure 2

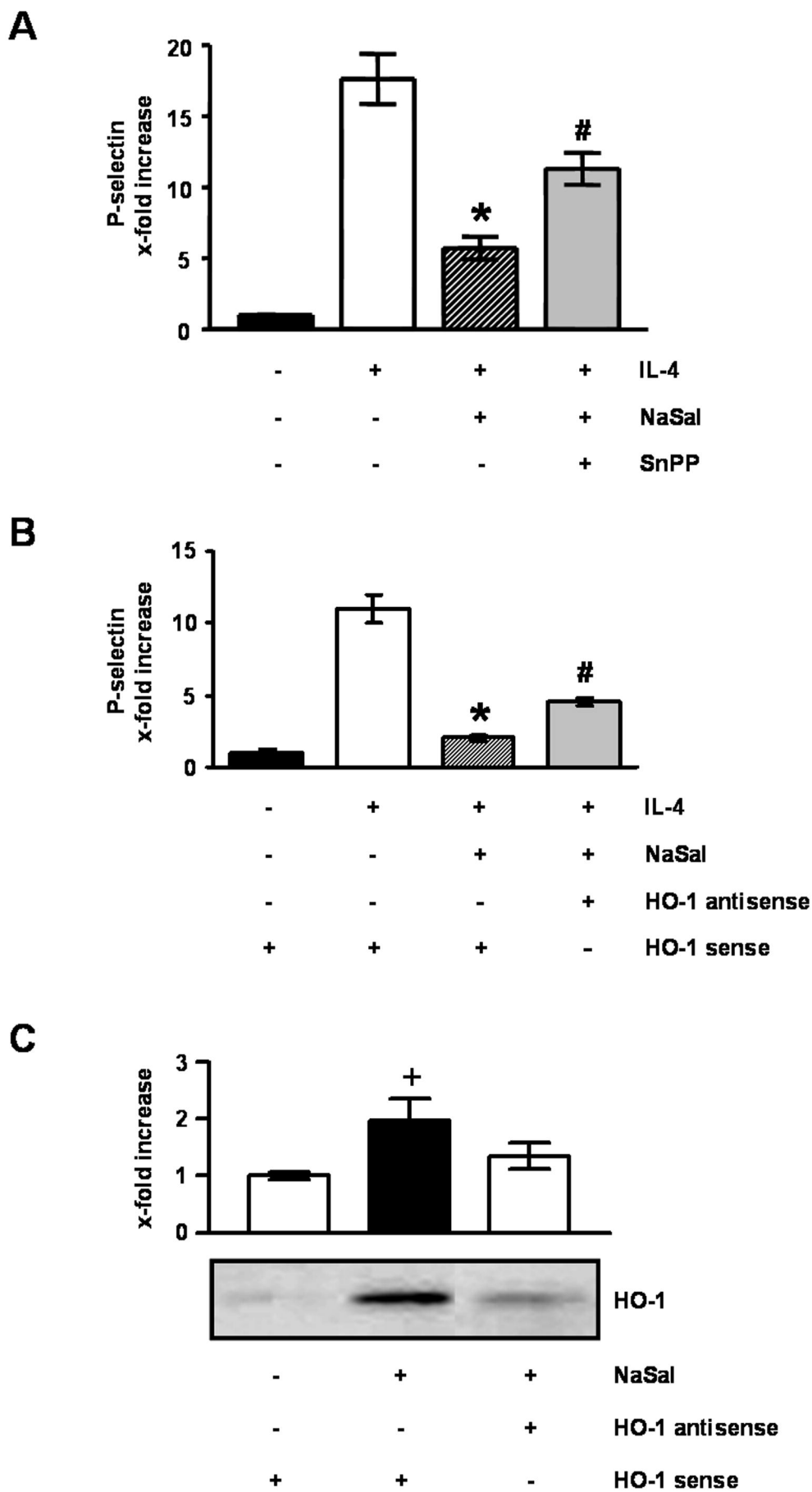
## A



## B



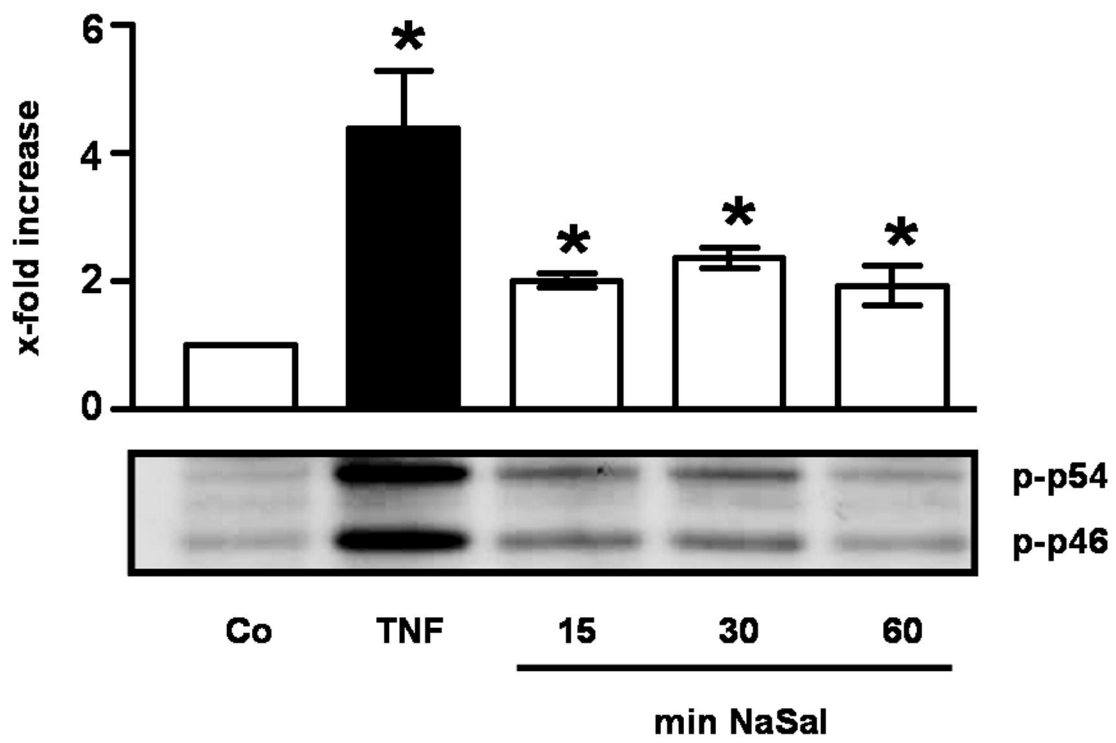
# Figure 3



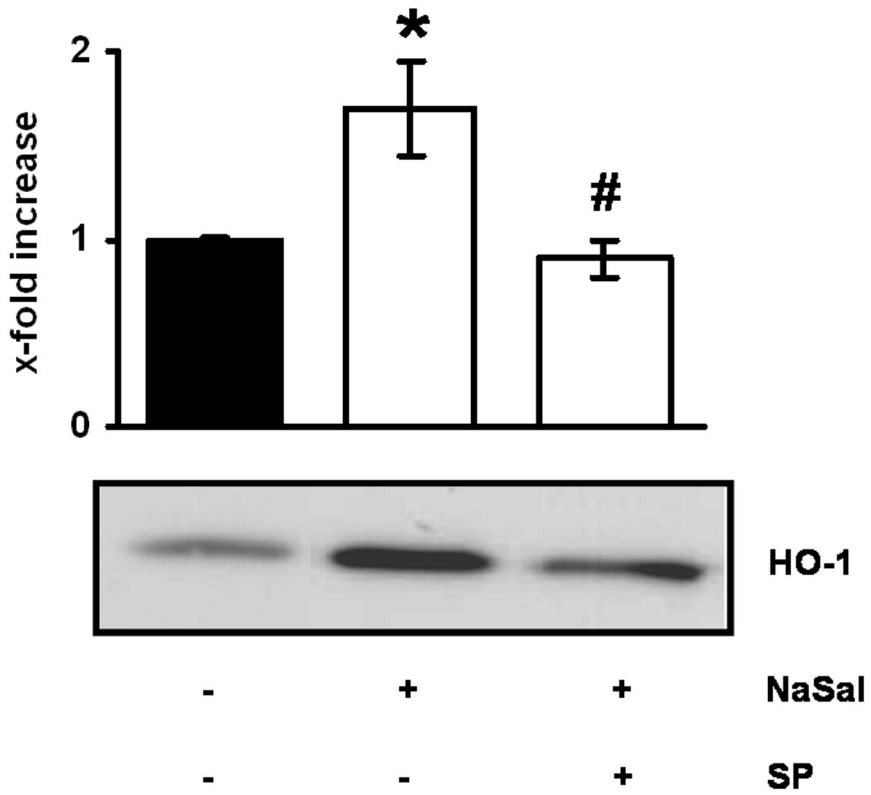


# Figure 4

**A**



**B**



# Figure 5

