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Antioxidants differentially affect nuclear factor κB-mediated nitric oxide synthase expression in vascular smooth muscle cells

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Abstract **Increased 'oxidative stress' resulting in the activation of nuclear factor κB (NF-κB) is thought to play a crucial role in the cytokine-mediated expression of the inducible isoform of nitric oxide synthase (iNOS) in different cell types. Therefore, the effects of four different antioxidants, carbocromen, chrysin, 3,4-dichloroisocoumarin (DCI) and *N*-acetylserotonin (NAS), on iNOS expression were investigated in vascular smooth muscle cells (VSMC). All antioxidants strongly reduced the phorbol ester-stimulated superoxide anion formation in native VSMC. Carbocromen (200 μM) and chrysin (50 μM) had no effect, while NAS (1 mM) abolished the increase in nitrine production and iNOS protein abundance in cultured VSMC exposed to interleukin-1β (IL-1β, 60 U/ml) or the adenylyl cyclase activator forskolin (10 μM). DCI also revealed a marked inhibitory effect in IL-1β-stimulated VSMC, but was less effective in cells treated with forskolin. DCI, but not NAS, also suppressed the activation of NF-κB in VSMC exposed to IL-1β, while no significant NF-κB activation was detected in forskolin-treated cells. These findings demonstrate that antioxidants differentially affect iNOS expression in VSMC both at the transcriptional level by preventing the activation of NF-κB and at the post-transcriptional level, presumably by promoting iNOS mRNA or protein degradation. They also suggest that reactive oxygen intermediates do not play a role in the activation of NF-κB by IL-1β in VSMC, and that transcripton factors other than NF-κB mediate the induction of iNOS expression by elevating the intracellular concentration of cyclic AMP.**

Key words: Nitric oxide synthase; Gene expression; NF-κB; Superoxide anion; Antioxidant; Vascular smooth muscle cell

1. Introduction

The high production of nitric oxide (NO) generated by the inducible isoform of NO synthase (iNOS), e.g. in macrophages and vascular smooth muscle cells (VSMC), can exert both protective and deleterious effects [1]. The isoenzyme-specific inhibition of iNOS expression and/or activity therefore represents an important therapeutic goal.

Changes in NO formation in iNOS-expressing cells are usually correlated with similar changes in iNOS mRNA abundance, indicating that a major part of iNOS regulation occurs at the level of transcription. The 5′-flanking region of the iNOS gene contains several bindings sites for *cis*-regulatory elements such as nuclear factor κB (NF-κB) [2–4], and activation of this transcription factor has been shown to be critical for iNOS gene expression in murine macrophages exposed to bacterial lipopolysaccharide (LPS) [5,6] and in interleukin-1β (IL-1β)-stimulated VSMC [7] and mesangial cells [8].

Since antioxidants are thought to inhibit the cytokine-mediated activation of NF-κB in many cell types [9], we have investigated whether these compounds also affect iNOS expression in rat aortic VSMC stimulated with IL-1β. In addition, we have studied their effects on iNOS expression in cells exposed to forskolin, since the induction of iNOS expression by agents which elevate the intracellular level of cAMP has been suggested to be mediated by transcription factor(s) other than NF-κB [8].

2. Materials and methods

2.1. Materials

3-β-Diethylaminoethyl-4-methyl-7-ethoxycarbonyl-methoxy-coumarin (carbochromen) was obtained from Hoechst; recombinant bovine superoxide dismutase from Grünenthal; *N*-acetylserotonin (NAS), chrysin (5,7-dihydroxyflavone), 3,4-dichloroisocoumarin (DCI), forskolin, xanthine oxidase and xanthine from Sigma; IL-1β from Collaborative Research; and Ro 31-8220 from Roche Products Ltd.

2.2. Determination of superoxide anion formation

Aortae were isolated from pentobarbitone-anesthetized male Wistar rats (250–300 g body weight), cleaned of adherent adipose and connective tissue, and cut into ring segments of approximately 7 mm in length. The endothelium was mechanically removed and superoxide anion (O_2^-) formation determined by monitoring the lucigenin (bis-*N*-methylacridinium nitrate)-enhanced chemiluminescence of O_2^- in a Lumac/3M model M2010 biocounter at 37°C. Briefly, the segments were incubated in 0.9 ml Hepes-buffered Tyrode solution, pH 7.35 (composition in mM: $CaCl_2$ 1.8, KCl 2.7, $MgCl_2$ 0.5, NaCl 137.0, NaH_2PO_4 0.36, D-glucose 5.0) in plastic cuvettes to which lucigenin was added at a concentration of 250 μM (final volume 1 ml). After monitoring basal O_2^- formation for 10 min, 1 μM phorbol 12-myristate 13-acetate (PMA) was added and the difference in chemiluminescence monitored for another 10 min. The assay was calibrated by monitoring the chemiluminescence signal of known amounts of O_2^- generated by xanthine oxidase (0.3–10 mU) and xanthine (50 μM). These had been previously determined spectrophotometrically by monitoring the reduction of ferricytochrome *c* [10]. O_2^- formation was calculated on the basis of the xanthine oxidase/xanthine calibration curve, the incubation time and the wet weight of the segments. The chemiluminescence assay was specific for O_2^-, no light emission was recorded in the presence of authentic NO or H_2O_2.

2.3. Cell culture

VSMC were isolated from the thoracic aorta of male Wistar-Kyoto rats (300–350 g body weight) by elastase/collagenase digestion and characterized by positive immunostaining with monoclonal antibodies raised against smooth muscle α-actin [11]. The cells were serially cultured in Waymouth medium (PAN Systems) containing non-essential amino acids (Biochrom), 100 U/ml of both penicillin and streptomycin, and 7.5% (v/v) fetal calf serum (PAN Systems). Upon reaching confluence they were passaged by using trypsin-EGTA (0.05/0.02%, w/v). All experiments were performed with cells from passages 8–16 seeded into 60 mm i.d. petri dishes (5×10^6 cells/dish) or 24-well plates ($3–5 \times 10^5$ cells/well).

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2.4. Experimental protocol

Confluent VSMC were incubated either in 0.3 ml (24-well plates) or 3 ml (60 mm Petri dishes) of serum-free Waymouth medium containing 0.1% (w/v) BSA in the absence (control cells) or presence of 60 U/ml IL-1β or 10 μM forskolin. When incubations were performed in the presence of the antioxidants, these were added together with IL-1β or forskolin. After 20 h in the incubator, the cells were harvested and the concentration of nitrite in the conditioned medium (200 μl plus 80 μl Griess reagent) was determined photometrically at 570 nm as described [12]. In some experiments, incubations were terminated after 30 min to prepare nuclear extracts [13]. Cell viability was assessed microscopically and by trypan blue exclusion.

2.5. Electrophoretic mobility shift analysis

Aliquots of nuclear protein (3 μg) were incubated with an NF-κB-specific oligonucleotide (5′-AGT TGA GGG GAC TTT CCC AGG C-3′, [14]; Promega) which was labelled with ^{32}P by random priming [15]. Binding experiments were performed with 1–4 μl nuclear extract, 3 μl 5 × binding buffer (50 mM Hepes, pH 7.5, 500 mM NaCl, 25% glycerol (w/v), 5 mM EDTA), 1 μg poly(dI-dC) as non-specific competitor DNA, and the oligonucleotide (5000 cpm) in a total volume of 15 μl for 30 min at ambient temperature. Non-denaturing polyacrylamide gel (4%) electrophoresis was performed with 1 × TBE buffer, pH 8.0 followed by autoradiography. To monitor the specificity of the binding reaction, the assay was performed in parallel with the same samples in the presence of a 100-fold excess of the non-labelled oligonucleotide. In some experiments, a supershift analysis was performed by preincubation of nuclear extracts from IL-1β-stimulated VSMC with 1 μg of a specific polyclonal anti-p65 (RelA) antibody (Santa Cruz Biotechnology) for 12 h at 4°C.

2.6. Western blot analysis

Immunoblot analysis of iNOS protein was performed essentially as described previously [16]. Protein extracts (10,000 × g supernatant) were separated by electrophoresis (20 μg protein/lane) on 8%-polyacrylamide gels in the presence of sodium dodecylsulfate and then transferred onto nitrocellulose membranes. The loading and transfer of equal amounts of protein in each lane was verified by staining of the protein bands with Ponceau S (0.2% in 3% trichloroacetic acid, v/v). After extensive washing with distilled water to remove the protein stain, the immobilized iNOS protein was visualized by subsequent incubation with a polyclonal anti-iNOS antibody (kindly provided by Dr. M. Marletta) and a secondary polyclonal peroxidase-conjugated anti-rabbit antibody (Amersham), followed by staining with the enhanced chemiluminescence (ECL) technique developed by Amersham. The antibody was specific for iNOS and did not cross-react with the constitutive NOS isoforms from rat brain or porcine aortic endothelial cells. The intensity of the iNOS-specific bands was quantified by densitometry (Pharmacia densitometer equipped with a Kappa CCD video camera and the ImageMaster software).

2.7. Statistical analysis

Unless indicated otherwise, all data in the figures and text are expressed as means ± S.E.M. of n experiments with different batches of VSMC. Statistical evaluation was performed by one-way analysis of variance followed by a Bonferroni t-test for multiple comparisons with a P-value <0.05 considered significant.

3. Results

3.1. Antioxidant activity

The antioxidative potential of the four antioxidants was tested with native VSMC in situ, i.e. in isolated de-endothelialized segments of the rat aorta, since O_2^- formation by cultured VSMC was frequently found to be below the level of detection of the chemiluminescence assay. Exposure of the segments to IL-1β (up to 100 U/ml) did not cause an appreciable increase in O_2^- formation for up to 30 min (not shown). Stimulation with PMA (1 μM), however, resulted in a marked increase in O_2^- formation which was virtually abolished by the protein kinase C (PKC) inhibitor Ro 31-8220 (1 μM; Fig. 1), suggesting that

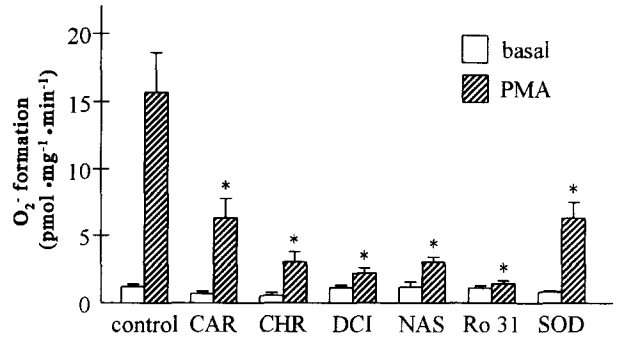

Fig. 1. Effects of carbocromen (CAR, 200 μM), chrysin (CHR, 50 μM), DCI (50 μM), NAS (1 mM), Ro 31-8220 (Ro 31, 1 μM) and superoxide dismutase (SOD, 30 nM) on the formation of O_2^- by native VSMC in situ in the absence and presence of PMA (1 μM) as indicated (n = 6–9, *P < 0.05 vs. control, i.e. VSMC not exposed to the aforementioned drugs).

the PMA-induced increase in O_2^- formation was mediated by the activation of PKC. Recombinant bovine superoxide dismutase (30 nM) also significantly reduced the PMA-dependent O_2^- formation, but was less effective than the PKC inhibitor (62% inhibition). Carbocromen (200 μM), chrysin (50 μM), DCI (50 μM) and NAS (1 mM) also strongly attenuated the PMA-induced increase in O_2^- formation (Fig. 1). Chrysin and NAS, but not DCI or carbocromen, also markedly reduced (>90% inhibition) the xanthine oxidase (10 mU)/xanthine (50 μM)-dependent generation of O_2^- (not shown).

3.2. Effects on nitrite production and iNOS protein expression

Exposure of the cultured VSMC to IL-1β (60 U/ml) caused a time-dependent increase in nitrite production (as an index of iNOS activity), iNOS mRNA and protein abundance which peaked at 18–20 h (cf. [17]). Actinomycin D (20 nM) and cycloheximide (10 μM) abolished the IL-1β-induced increase in nitrite production and iNOS protein abundance (not shown), confirming that the induction of iNOS gene expression by IL-1β in VSMC requires both DNA transcription and de novo protein synthesis.

Co-incubation of the cells with carbocromen (up to 200 μM) or chrysin (up to 50 μM) did not affect the IL-1β-induced increase in nitrite production (Fig. 2a) or iNOS protein abundance (not shown). Higher concentrations of these two antioxidants could not be employed due to their cytotoxic effects. DCI and NAS, on the other hand, suppressed the IL-1β-induced increase in nitrite production and iNOS protein abundance with IC_{50} values of 10 μM (Fig. 2a) and 0.5 mM (cf. [18]), respectively. In some experiments, DCI and NAS also attenuated the basal level of nitrite production and iNOS protein expression in unstimulated VSMC (not shown).

Treatment of the cells with forskolin (10 μM) also resulted in a prominent increase in nitrite production and iNOS protein expression (Fig. 2b), the magnitude of which was comparable to the effects exerted by IL-1β. Co-incubation with NAS, but not with carbocromen or chrysin (not shown), virtually abolished the stimulatory effect of forskolin on nitrite production and iNOS protein expression (Fig. 2b). When compared to IL-1β-stimulated VSMC, the inhibitory effect of DCI was much less pronounced in forskolin-treated cells (88% and 41% decrease in nitrite production respectively).

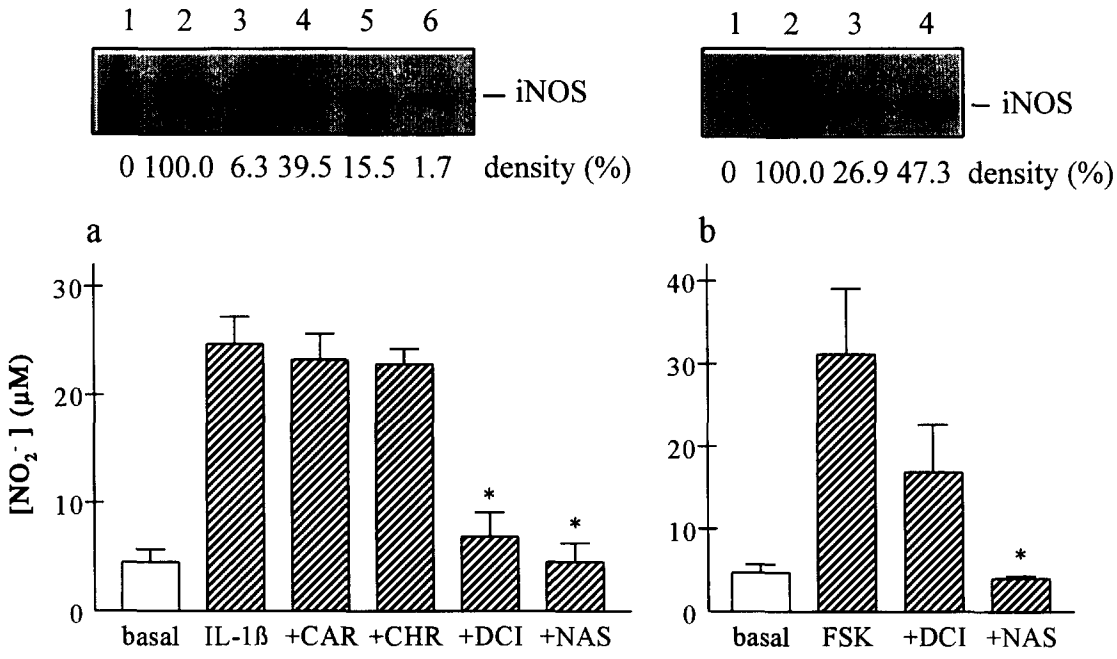

Fig. 2. (a) Effects of carbocromen (+CAR, 200 μM), chrysin (+CHR, 50 μM), DCI (50 μM) and NAS (1 mM) on the increase in nitrite production in cultured VSMC exposed to IL-1β (60 U/ml) for 20 h. 'Basal' denotes the nitrite level in the conditioned medium of VSMC not exposed to IL-1β (n = 5–8, *P < 0.05). (b) Effects of DCI (50 μM) and NAS (1 mM) on the increase in nitrite production in cultured VSMC exposed to forskolin (FSK, 10 μM) for 20 h (n = 4–5, *P < 0.05). The immunoblots above the bar charts depict the effects of DCI and NAS on the increase in iNOS protein abundance in cultured VSMC exposed to (a) IL-1β (lane 1, control; lane 2, IL-1β; lane 3, IL-1β + 1 mM NAS; lane 4, IL-1β + 10 μM DCI; lane 5, IL-1β + 25 μM DCI; lane 6, IL-1β + 50 μM DCI) or (b) forskolin (lane 1, control; lane 2, forskolin; lane 3, forskolin + 1 mM NAS; lane 4, forskolin + 50 μM DCI). They are representative of four experiments with different batches of VSMC performed in the same manner.

NAS is a potent sepiapterin reductase inhibitor [19] and its effect on iNOS expression may be related to a lack of tetrahydrobiopterin (BH_4), which is an essential co-factor for NO biosynthesis [19–22]. Dihydrobiopterin (BH_2) is a substrate for BH_4 synthesis even under conditions where sepiapterin reductase is blocked [19]. To test the hypothesis that NAS blocks iNOS expression by limiting the availability of BH_4, VSMC were incubated with IL-1β and BH_2 (0.3 mM), in the absence and presence of NAS. BH_2 (0.3 mM), however, did not affect nitrite production (Fig. 3) and iNOS protein expression (not shown) in IL-1β-stimulated VSMC and failed to reverse the inhibitory effects of NAS.

3.3. Effects on NF-κB activation

Two NF-κB-specific DNA-protein complexes were identified in nuclear extracts of the cultured VSMC (Fig. 4). While the faster migrating complex was also present in extracts of control cells, the slower migrating complex was detectable only in extracts from IL-1β-stimulated cells. Treatment of these extracts with a specific anti-p65 antibody resulted in a supershift of the slower migrating DNA-protein complex, suggesting that this complex comprises the p65/p50 heterodimer of NF-κB. Consequently, the faster migrating DNA-protein complex is likely to represent the p50/p50 homodimer which has been previously demonstrated to be present in nuclear extracts of VSMC [27]. Moreover, by Western blot analysis we also detected an increase in p65 protein in nuclear extracts of IL-1β-stimulated VSMC, thus confirming the activation of NF-κB (not shown).

Neither the p65-containing DNA-protein complex nor the p50/p50-like complex were detectable in nuclear extracts from DCI (50 μM)-treated VSMC, suggesting that the coumarin derivative abolished both the basal and IL-1β-mediated activation of NF-κB. NAS had no significant effect, while the abundance of both complexes was increased rather than decreased in nuclear extracts of VSMC stimulated with IL-1β in the presence of carbocromen or chrysin (Fig. 4).

Treatment of the cells with forskolin had either no effect on the intensity of the two NF-κB specific DNA-protein complexes or weakly enhanced that of the p65/p50 heterodimer; the

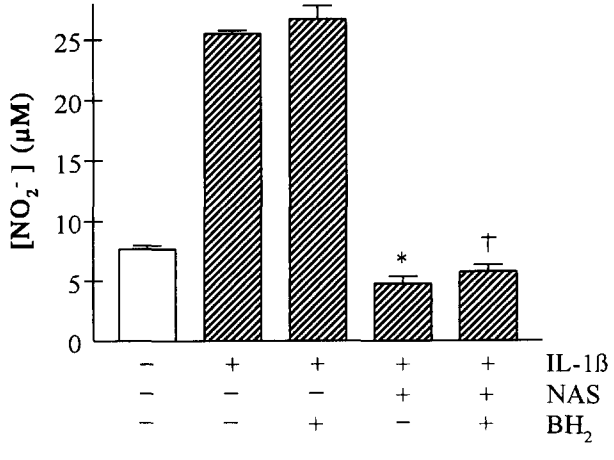

Fig. 3. Effects of dihydrobiopterin (BH_2, 0.3 mM) on nitrite production in cultured VSMC exposed to IL-1β (60 U/ml) for 20 h in the absence or presence of NAS (1 mM) as indicated (n = 3; *P < 0.05 vs. IL-1β, †P < 0.05 vs. IL-1β plus BH_2).

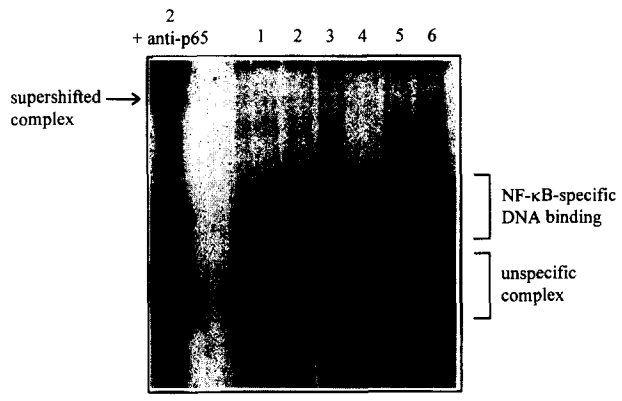

Fig. 4. Effects of carbocromen (200 μM, lane 6), chrysin (50 μM, lane 5), DCI (50 μM, lane 4) and NAS (1 mM, lane 3) on the DNA-binding activity of NF-κB in nuclear extracts from VSMC exposed to IL-1β (60 U/ml, lane 2) for 30 min (control, lane 1). The gel shift shown is representative of five experiments with different batches of VSMC performed in the same manner; it also shows the supershift of the slower migrating NF-κB-specific band with the anti-p65 antibody.

latter effect was reversed in VSMC treated with forskolin and DCI (not shown).

4. Discussion

The present findings suggest that antioxidants per se have no effect on iNOS expression in VSMC. All four test compounds strongly diminished the phorbol ester-stimulated, PKC-dependent formation of O_2^- in native VSMC in situ. Chrysin and NAS also effectively scavenged O_2^- generated by xanthine oxidase in vitro. However, only DCI and NAS significantly inhibited iNOS protein expression in cultured VSMC exposed to IL-1β. Moreover, only DCI inhibited the IL-1β-induced activation of NF-κB. Although these findings confirm that NF-κB activation represents a crucial step in the induction of iNOS gene expression in IL-1β-stimulated VSMC [7], they also suggest that reactive oxygen intermediates do not play a role in the activation of NF-κB in these cells.

One could argue that the antioxidants employed in the present study are not capable of scavenging reactive oxygen species inside the cell and therefore do not block the activation of NF-κB. When compared to SOD, which can scavenge O_2^- only outside the cell, three of the test compounds (chrysin, DCI and NAS) were clearly more effective in inhibiting the PMA-induced formation of O_2^-, suggesting that these antioxidants are also capable of scavenging intracellular O_2^-. This notion is supported by the finding that lucigenin is taken up by VSMC [24] and emits light upon reaction with intracellular O_2^- [25]. Another argument is that the antioxidants prevent the PMA-induced formation of O_2^-, because they interfere with the activation of PKC. This possibility, however, is unlikely for the following reasons: (i) chrysin and NAS scavenged xanthine oxidase-derived O_2^-; (ii) PMA caused an activation of NF-κB in cultured VSMC which was not blocked by chrysin (Hecker et al., unpublished observation); and (iii) none of the four antioxidants significantly affected the activity of a semi-purified PKC isolated from rat brain (Crause et al., unpublished observation).

As previously shown for mesangial cells [8], an increase in intracellular cAMP alone also causes an expression of the iNOS gene in VSMC. Moreover, the partial inhibition by DCI of the forskolin-induced increase in nitrite production and iNOS protein abundance suggested that in contrast to mesangial cells NF-κB activation may play a role in the cAMP-mediated increase in iNOS expression in VSMC. However, no significant activation of the transcription factor was detected in forskolin-treated VSMC, and DCI was clearly less effective in inhibiting iNOS expression in VSMC exposed to forskolin as compared to IL-1β-treated cells. It is thus unlikely that NF-κB activation plays a major role in the cAMP-mediated increase in iNOS expression in VSMC. On the other hand, DCI may affect the activation of other transcription factors in response to the rise in intracellular cAMP elicited by forskolin.

DCI is a potent serine protease inhibitor and this class of compounds has been shown to inhibit NF-κB activation by preventing the proteolytic degradation of the inhibitory IκB subunit [26]. It is not clear, however, whether this effect is achieved by preventing the targeting of IκB to the proteasome or its degradation by the proteasome, since serine protease inhibitors have also been shown to block the phosphorylation of IκB which is crucial for its targeting to the proteasome [27]. Irrespective of the precise mechanism of action by which DCI prevented the activation of NF-κB in VSMC exposed to IL-1β, this effect is unlikely to be related to its antioxidative properties.

The finding that NAS did not affect NF-κB activation in VSMC points to an effect at the post-transcriptional level, presumably involving iNOS mRNA or protein stability. NAS is a potent sepiapterin reductase inhibitor and its effect on iNOS expression in macrophages,VSMC and mesangial cells has been associated with an inhibition of BH_4 synthesis [19–22]. BH_4 can be synthesized both by the de novo pathway via GTP cyclohydrolase and by the salvage pathway involving sepiapterin reductase. It is not clear, however, whether the availability of BH_4 is rate-limiting both for NO synthesis and iNOS expression in cytokine-stimulated VSMC [19,22]. To test this hypothesis, the effect of BH_2 on the inhibition by NAS of iNOS protein expression was investigated, since BH_2 is a substrate for BH_4 synthesis irrespective of a blockade of sepiapterin reductase by NAS [19]. As in LPS/interferon-γ-stimulated murine macrophages [18], BH_2 failed to reverse the inhibitory effect of NAS on iNOS protein expression in IL-1β-stimulated VSMC, suggesting that this effect of NAS is unrelated to the availability of BH_4. This notion is also supported by the finding that the IC_{50} value of NAS for the inhibition of iNOS protein expression in VSMC is 0.5 mM [18], while the halfmaximal concentration of NAS required to inhibit sepiapterin reductase activity in rat erythrocyte lysates is much lower, i.e. 0.8 μM (Hecker et al., unpublished observation). Moreover, carbocromen, which is almost as effective as NAS in inhibiting sepiapterin reductase (IC_{50} 4.0 μM), had no effect on iNOS protein expression in VSMC exposed to IL-1β or forskolin. The precise mechanism of action by which NAS inhibits iNOS protein expression in activated VSMC thus remains to be elucidated.

In summary, the present findings show that only certain antioxidants inhibit iNOS expression in cultured VSMC either by preventing the activation of NF-κB or by promoting the degradation of iNOS mRNA or protein. This diversity in the efficacy and mechanism of action of this class of compounds suggests that the activation of NF-κB by IL-1β in VSMC does not involve a redox-sensitive step.

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