Quinazoline derivatives suppress nitric oxide production by macrophages through inhibition of NOS II gene expression

Noriko Fujiwara[a], Ayako Okado[a], Han Geuk Seo[a], Junichi Fujii[a], Kigen Kondo[b], Naoyuki Taniguchi[a,*]

[a]*Department of Biochemistry, Osaka University Medical School, 2-2 Yamadaoka, Suita, Osaka 565, Japan*
[b]*Minase Research Institute, Ono Pharmaceutical Co., Ltd., 3-1-1 Sakurai, Shimamoto, Mishima, Osaka 618, Japan*

Received 29 July 1996; revised version received 6 September 1996

Abstract We have found three novel quinazolidine derivatives which inhibit the formation of nitrite dose-dependently in a murine macrophage cell line, RAW264.7. The decreased nitrite formation was due not to the inhibition of nitric oxide synthase activity but to suppression of NOS II mRNA and protein expression. In rat vascular smooth muscle cells (VSMC), however, these compounds rather enhanced NOS II mRNA. These compounds also prevented LPS-stimulated heme oxygenase-1 (HO-1) and cyclooxygenase-2 (COX-2) gene expression in RAW264.7 cells, but again not in VSMC. The three quinazolidine derivatives specifically inhibit gene expression of NOS II, HO-1 and COX-2 only in macrophage cells, indicating that they are selective inhibitors of inducible gene expression in macrophages.

Key words: Nitric oxide; Nitric oxide synthase; Cyclooxygenase; Heme oxygenase; Gene expression

1. Introduction

Many types of cells produce nitric oxide (NO) by oxidizing L-arginine with molecular oxygen [1]. Three NO synthase (NOS) isoforms have been identified and cloned [2]. One form, constitutively expressed mainly in the brain (NOS I), is Ca^{2+}-dependent, but the inducible form (NOS II) found in macrophages, smooth muscle cells and hepatocytes is Ca^{2+}-independent. The other form of NOS (NOS III), associated with membranes, is expressed mainly in endothelial cells. NO produced by NOS III is essential for controlling endothelial functions in the cardiovascular system. NOS I functions in neuronal transmission under physiological conditions. On the other hand, macrophages and smooth muscle cells activated by cytokines and lipopolysaccharide (LPS) release a large amount of NO, which plays a key role in the pathophysiology of a variety of diseases, including sepsis and inflammation.

Because NO is produced by NOS II in large amounts and damages tissues, inhibitors which are specific to NOS II but do not affect other isoforms are demanded. N^{ω}-Monomethyl-L-arginine (L-NMMA), aminoguanidine and *S*-ethylisothiourea are potent inhibitors of NOS II activity [3–5]. However, most NOS inhibitors including three were designed to mimic the substrate L-arginine or the product L-citrulline, and are therefore not totally specific for NOS II. On the other hand, it has been confirmed that glucocorticoids and transforming growth factor (TGF)-β1 inhibited NOS II expression in many cellular systems [6,7]. Thus it may become a useful approach to establish compounds which inhibit the induction of a specific gene(s) during inflammation.

Here we report three novel compounds which inhibit the nitrite formation in a murine macrophage cell line, RAW264.7, without affecting NOS II activity. The mechanism is likely to be related to the inhibition of NOS II gene transcription in RAW264.7 cells. Moreover, they prevented the LPS-stimulated HO-1 and COX-2 mRNA expression in RAW264.7 cells, whereas they enhanced NOS II and COX-2 induction and had no effect on HO-1 mRNA expression in VSMC.

2. Materials and methods

L-[U-^{14}C]Arginine monohydrochloride (319 mCi/mmol) and [α-^{32}P]dCTP were obtained from Amersham Corp. NADPH was obtained from Oriental Yeast. Tetrahydrobiopterin was from Wako Pure Chemical Industries. Dowex 50W-X8 was obtained from Muromachi Kagaku Kogyo. A monoclonal antibody raised against mouse NOS II was obtained from Transduction Laboratories. Recombinant IL-1β (specific activity 20 U/ng protein) was kindly supplied by Otsuka Pharmaceutical Co., Ltd. Other reagents were of the highest grade available.

2.1. Assaying of nitrite produced by RAW264.7 cells

RAW264.7 cells, a mouse macrophage-like cell line transformed with the Abelson leukemia virus, obtained from the American Type Culture Collection, were cultured in Dulbecco's modified Eagle's medium, containing 4.5 g/l glucose, 100 units/ml of penicillin and 100 μg/ml of streptomycin, supplemented with 10% heat-inactivated fetal bovine serum (Gibco/BRL) at 37°C under an atmosphere of 95% air and 5% CO_2. 5×10^5 cells/ml/well of 12-well plates were incubated for 18 h in medium supplemented with 10 ng/ml LPS and varying concentrations of a compound dissolved in dimethylsulfoxide. In control experiments, 0.05% dimethylsulfoxide was always included as a vehicle. Following incubation, nitrite in the medium was quantitated using the Griess reagent as described [8]. This method, which detects only nitrite in the medium, was not affected by compounds used in this work.

2.2. Assaying of nitrite produced by VSMC

VSMC isolated from rat aorta were cultured in Dulbecco's modified Eagle's medium, containing 100 units/ml of penicillin and 100 μg/ml of streptomycin, supplemented with 20% heat-inactivated fetal bovine serum (Gibco/BRL) at 37°C under an atmosphere of 95% air and 5%

*Corresponding author. Fax: (81) (6) 879-3429.
E-mail: proftani@biochem.med.osaka-u.ac.jp

Abbreviations: NO, nitric oxide; NOS, NO synthase; NOS I, brain NOS; NOS II, macrophage NOS; NOS III, endothelial NOS; DIQ, 4-(1,1-dimethyl-1,2-methoxy-ethylamino)-2-(imidazol-1-yl)-quinazoline dihydrochloride; IMT, 2-(imidazol-1-yl)-4-(2-methoxyethylamino)-5,6,7,8-tetrahydroquinazoline dihydrochloride; IPE, 2-{2-[2-(imidazol-1-yl)-5-methylthieno[2,3-d]pyrimidine-4-ylamino]ethoxy}ethanol dihydrochloride; LPS, lipopolysaccharide; L-NMMA, N^{ω}-monomethyl-L-arginine; TBS, Tris-buffered saline; VSMC, vascular smooth muscle cells; SSC, standard saline citrate; IL-1β, interleukin-1β

PII S0014-5793(96)01058-7

CO_2. 1×10^5 cells/ml/well of 12-well plates were incubated for 24 h with 10 ng/ml IL-1β and a compound. After incubation, nitrite was quantitated.

2.3. Assaying of NOS activity

After stimulation with 10 ng/ml LPS for 24 h, RAW264.7 cells were sonicated and the supernatant was used for the NOS assay. NOS activity was determined by assessing the conversion of L-[U-^{14}C]arginine to L-[U-^{14}C]citrulline in the presence of 1 mM EGTA by the method described previously [9].

2.4. RNA preparation and Northern analysis

After incubation with LPS or IL-1β in the presence of an inhibitor, total RNA was prepared from RAW264.7 cells and VSMC in 10-cm dishes [10], and quantitated by measuring the absorbance at 260 nm. 20 μg of total RNA was electrophoresed on an 1% agarose gel containing 2.2 M formaldehyde, transferred to a Zeta-Probe membrane, and then hybridized with a ^{32}P-labeled NOS II cDNA probe [11] at 42°C in the presence of 50% formamide. Then it was washed twice at 55°C with 2×standard saline citrate (SSC) and 0.1% SDS for 30 min, and then twice at high stringency with 0.3×SSC and 0.1% SDS for 30 min at 65°C. Kodak XAR films were exposed for 1–3 days with an intensifying screen at −80°C. The blots were stripped by boiling in 0.1% SDS and 0.1×SSC, and reprobed with COX-2 [12] and HO-1 [13] cDNA probes serially using the hybridization and washing conditions given above.

2.5. Western blot analysis

Protein from RAW264.7 cells (20 μg) incubated with 10 ng/ml LPS in the presence of an inhibitor was subjected to 7.5% SDS-polyacrylamide gel electrophoresis and then transferred to a nitrocellulose membrane under semi-dry conditions with the use of a Semiphor TE-70 semi-dry transfer unit (Hoefer). After blocking by incubation with 4% skim milk in TBS for 12 h at 4°C, the membrane was washed four times with TBS and 0.05% Tween 20, and then incubated with an antibody against mouse NOS II at a 1:2500 dilution for 2 h. After washing, the membrane was incubated with peroxidase-conjugated goat anti-mouse IgG (Organon Teknika Corp.) for 2 h. A chemiluminescence method involving an ECL kit (Amersham) was employed to detect the peroxidase activity.

3. Results

We have screened a series of compounds, which were developed as inhibitors of phosphodiesterase types IV and V, by assessing their inhibitory effects on nitrite production by LPS-stimulated RAW264.7 cells. On this screening, we found that three novel quinazolidine derivatives, 4-(1,1-dimethyl-1,2-methoxy-ethylamino)-2-(imidazol-1-yl)-quinazoline dihydrochloride (DIQ), 2-(imidazol-1-yl)-4-(2-methoxyethylamino)-5,6,7,8-tetrahydroquinazoline dihydrochloride (IMT), and 2-{2-[2-(imidazol-1-yl)-5-methylthieno[2,3-d]pyrimidine-4-ylamino]ethoxy}ethanol dihydrochloride (IPE), effectively inhibited nitrite production. Their chemical structures are shown in Fig. 1. These compounds at 50 μM did not affect cellular viability, as assessed by monitoring the mitochondrial reduction of tetrazolium to formazan (data not shown), indicating that they were not cytotoxic at these concentrations.

The effects of these compounds and a widely used inhibitor, L-NMMA, on LPS-induced nitrite production by RAW264.7 cells are shown in Fig. 2. Essentially the same inhibition patterns were observed when the cells were stimulated with interferon-γ (data not shown). All three compounds inhibited nitrite production dose-dependently. DIQ and IPE inhibited more than 95% of the nitrite production at 50 μM. However, the inhibitory effects did not essentially change when they were administered at 4 h after stimulation with LPS, while they exhibited significantly reduced inhibitory effects when administered at 12 h after LPS treatment (data not shown). The extent of inhibition by L-NMMA remained the same and did not depend on the time after administration.

To determine whether or not the inhibition of nitrite production was due to the direct action of these compounds on the NOS II enzyme, we examined their effects on L-[U-^{14}C]citrulline formation from L-[U-^{14}C]arginine using cellular extracts of LPS-stimulated RAW264.7 cells. These compounds had no substantial effect on the NOS II activity in a cell-free system, while L-NMMA inhibited NOS II activity dose-dependently, as reported, at all concentrations (data not shown), indicating that the target molecule of these compounds was not the NOS II protein itself.

Since the data described above suggest that the inhibition of NO production by RAW264.7 cells occurs at the level of NOS II expression, we examined whether or not they inhibited NOS II mRNA expression by Northern blot analysis. As shown in Fig. 3, they suppressed the level of NOS II mRNA in RAW264.7 cells in a dose-dependent manner. However, the extent of the inhibition was low compared to in the control and was not totally consistent with that of nitrite production, as shown in Fig. 2. Thus, the inhibitory effects of these compounds on nitrite production cannot be explained solely by inhibition of transcription of the NOS II gene. Then we examined the level of the NOS II protein in RAW264.7 cells at 24 h after stimulation with LPS in the presence of 5 or 50 μM each inhibitor. Immunoblot analysis indicated that the levels of NOS II protein were suppressed to a similar extent to NOS II mRNA in the presence of the inhibitors (Fig. 4). Taken together, all these data suggest

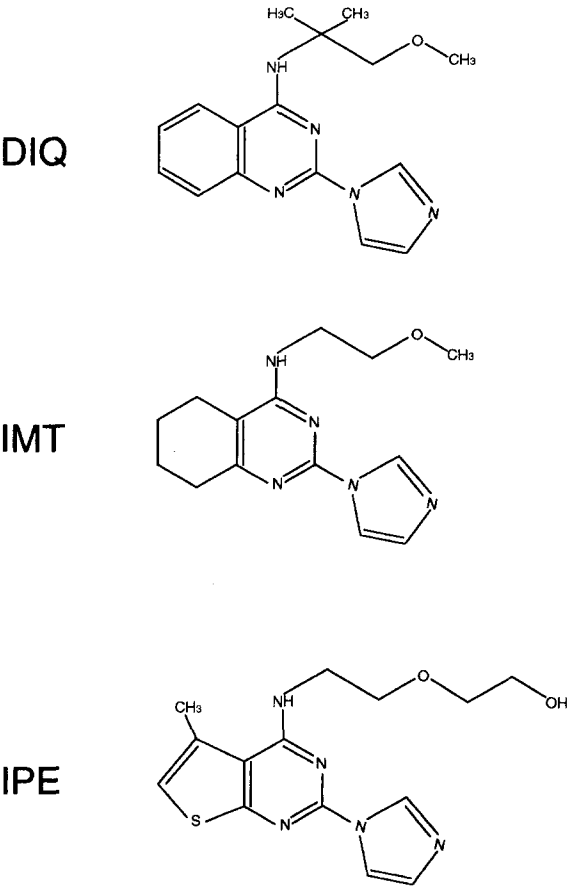

Fig. 1. Structures of the compounds used.

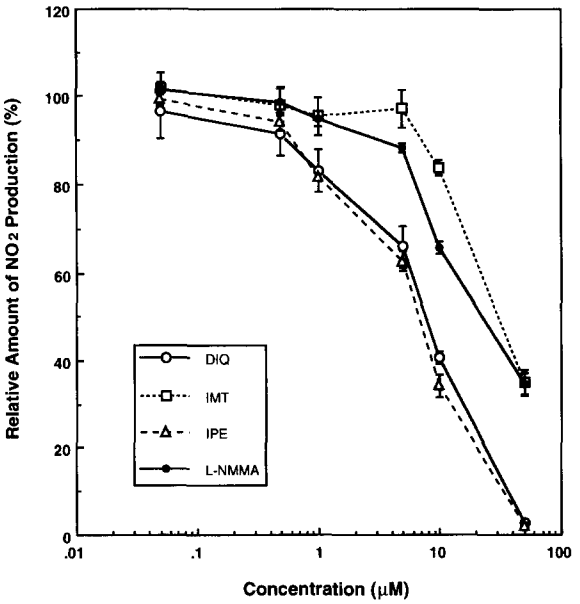

Fig. 2. Dose-response curves of nitrite production by RAW264.7 cells stimulated with LPS. RAW264.7 cells, 5×10^5/ml/well, were incubated with 10 ng/ml LPS in the presence of varying concentrations of inhibitors for 18 h. Nitrite in the conditioned medium was quantitated, that in the control experiment being taken as 100%. Each point indicates the mean ± S.D. for triplicate experiments.

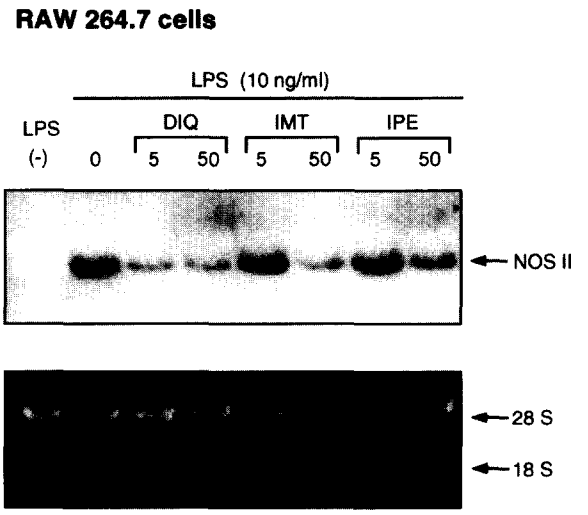

Fig. 3. Effects of inhibitors on NOS II mRNA expression in RAW264.7 cells stimulated with LPS. Total RNA was extracted from RAW264.7 cells stimulated with LPS in the presence of inhibitors (5 or 50 μM) for 18 h. Northern blot analysis was carried out using a mouse NOS II cDNA fragment as a probe.

that these reagents inhibited nitrite production through suppression of NOS II gene expression in RAW264.7 cells.

We also examined the effects of these inhibitors on the nitrite production in VSMC, which are another type of cells expressing NOS II by IL-1β. As shown Fig. 5, their effects on the nitrite production in VSMC were complicated because they did not inhibit or only slightly enhanced nitrite production at low concentrations, but inhibited it at higher concentrations. Expression of NOS II mRNA was enhanced dose-dependently by them. We studied the effects of three inhibitors on the expression of another two inflammation-associated enzymes in RAW264.7 cells and VSMC to determine whether or not the effects were specific to the NOS II gene. As shown in Fig. 6, they inhibited HO-1 and COX-2 mRNA in RAW264.7 cells in the same manner as the NOS II gene, but enhanced COX-2 mRNA expression in VSMC, as found for NOS II mRNA. However, they had no effect on HO-1 mRNA in VSMC except for IMT, which slightly enhanced HO-1 mRNA at 50 μM.

4. Discussion

We found three novel compounds which inhibited NO production by macrophages stimulated with interferon-γ and LPS, and investigated the mechanism underlying the inhibition. These compounds significantly inhibited nitrite production at micromolar concentrations in RAW264.7 cells, but had no direct effect on the NOS activity, as judged by assaying the conversion of L-arginine to L-citrulline. They partially reduced the level of NOS II mRNA as well as that of protein. Thus it was likely that these compounds regulated the NOS II expression at the level of gene induction.

Recent studies have shown that the promoter region of the NOS II gene contains numerous potential sites for the binding of a number of different transcription factors [14]. A key region of the promoter in mediation of the response to LPS is downstream of the NF-κB site [15] and an IRF-1 binding site in macrophages stimulated by IFN-γ or LPS [16]. On the other hand, it was reported that an upstream NF-κB site is of key importance in mediating the synergistic effect of cytokines on NOS II gene activity within VSMC [17]. We examined the effects of these compounds on the binding of NF-κB to the reported responsive elements in the gene by means of a gel shift assay, but they did not affect the binding (data not shown).

On the other hand, we obtained the opposite results for VSMC, i.e. the inhibitors enhanced nitrite production and NOS II mRNA. The discrepancy between nitrite production and NOS II mRNA levels may be explained as follows. While nitrite accumulates in the medium during incubation, NOS II mRNA turns over rapidly. In addition, when we observed effects of these compounds on the levels of NOS II mRNA

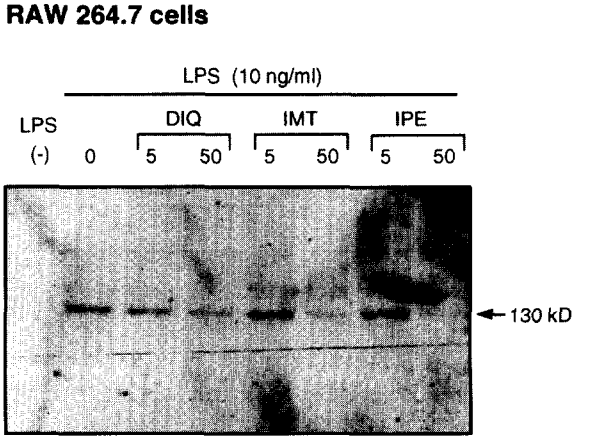

Fig. 4. Effects of inhibitors on NOS II protein expression. RAW264.7 cells were stimulated under the same conditions as in Fig. 3 for 24 h. After sonication and centrifugation, 20 μg of the soluble protein from the cells was analyzed by Western blotting using an anti-mouse NOS II antibody.

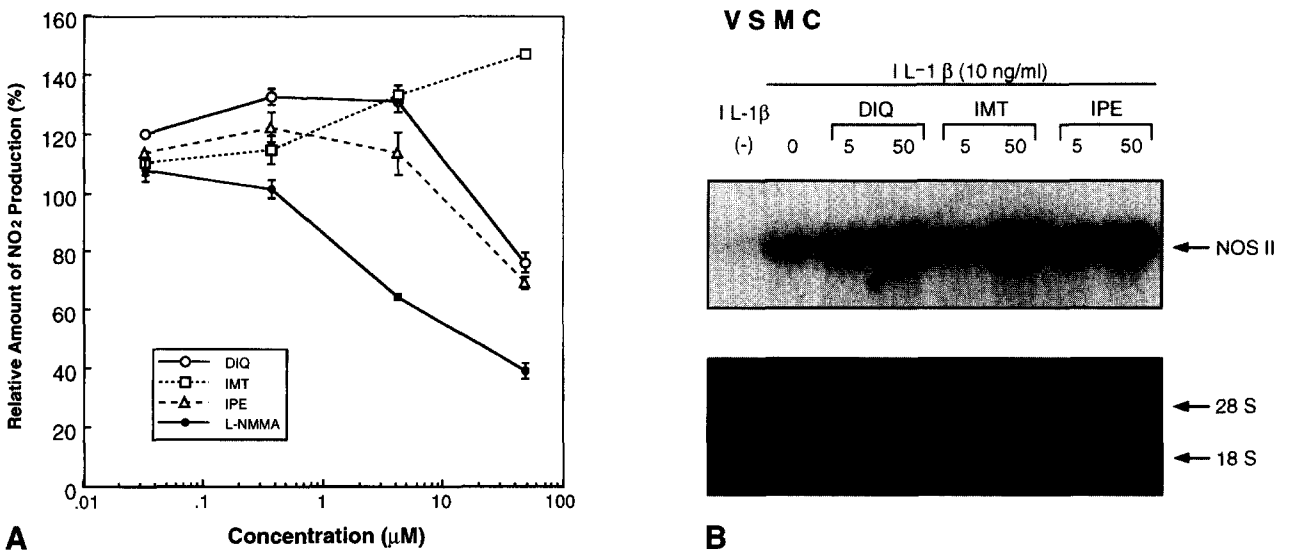

Fig. 5. A: Effects of the inhibitors on nitrite production in VSMC stimulated with IL-1β. VSMC, 1×10^5 cells/ml/well, were incubated with 10 ng/ml IL-1β in the presence of varying concentrations of inhibitors for 24 h. Nitrite in the conditioned medium was quantitated, that in the control experiment being taken as 100%. Each point indicates the mean ± S.D. for triplicate experiments. B: Effects of inhibitors on NOS II mRNA expression in VSMC stimulated with IL-1β. Northern blot analysis of total RNA prepared from VSMC under the same conditions as in A was carried out as described in the legend to Fig. 3.

at different time points, more mRNA accumulated at 24 h than at shorter incubation times (data not shown). We recently obtained similar results showing that high levels of cAMP prevented the induction of NOS II mRNA and NO production in macrophages, but enhanced NOS II mRNA and NO formation in VSMC (manuscript in preparation). Since the inhibitors used in this study were phosphodiesterase types IV and V inhibitors, in order to examine the possibility that the cAMP level may be altered by the inhibitors, we measured the cAMP contents 30 min after treatment with the inhibitors. However, no change was observed in either RAW264.7 cells or VSMC (data not shown).

For further characterization of their action, we examined the expression of two other genes involved in inflammation. HO catalyzes the first and rate-limiting step in the oxidative degradation of heme to bilirubin [18]. Among the two isoforms, HO-1 is induced by inflammatory cytokines, LPS and other stimulants. COX is the enzyme which converts arachidonic acid to prostaglandins. COX-2 but not COX-1 is induced in a number of cells by proinflammatory stimuli and is involved in various types of inflammation. The inhibitors suppressed the expression of all these enzymes in macrophages, but increased COX-2 mRNA expression in VSMC. They had no effect on the expression of HO-1 in VSMC. The functions of these inhibitors are likely to be related to the transcription of the genes, although we could not identify the target molecule involved in their action. Identification of the target molecule would also provide us with useful information about the differential responses of these inducible genes in macrophages and VSMC. Moreover, these quinazoline derivatives may shed new insight for the development of selective inhibitors of inducible gene expression under inflammation in tissues.

Acknowledgements: This work was supported in parts by Grants-in-Aid from the Ministry of Health and Welfare, Japan.

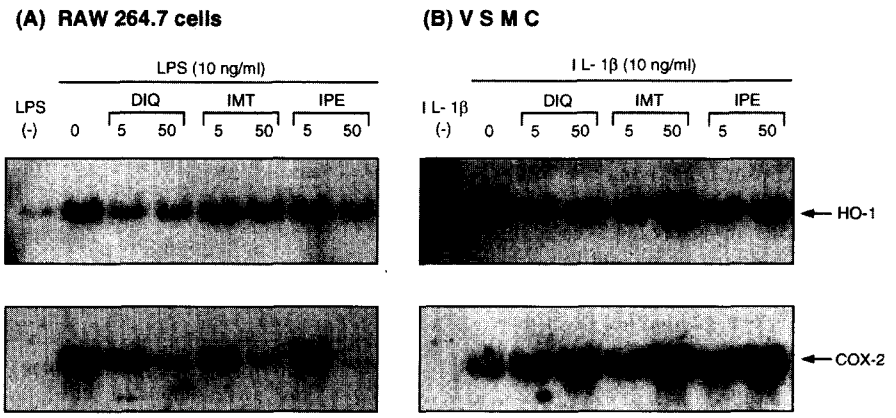

Fig. 6. Effects of inhibitors on HO-1 and COX-2 expression in RAW264.7 cells and VSMC. The same membranes as in Figs. 3 and 5B were dehybridized and then rehybridized with HO-1 and COX-2 cDNAs as probes.

References

[1] Moncada, S., Palmer, R.M.J. and Higgs, E.A. (1991) Pharmacol. Rev. 43, 109–142.
[2] Marletta, M.A. (1993) J. Biol. Chem. 268, 12231–12234.
[3] Rees, D.D., Palmer, R.M.J., Hodson, H.F. and Moncada, S. (1989) Br. J. Pharmacol. 96, 418–424.
[4] Corbett, J.A., Tilton, R.G., Chang, K., Hasan, K.S., Ido, Y., Wang, J.L. Sweetland, M.A., Lancaster, J.R., Jr., Williamson, J.R. and McDaniel, M.L. (1992) Diabetes 41, 552–556.
[5] Garvey, E.P., Oplinger, J.A., Tanoury, G.J., Sherman, P.A., Fowler, M., Marshall, S., Harmon, M.F., Paith, J.E. and Furfine, E.S. (1994) J. Biol. Chem. 269, 26669–26676.
[6] Dilrosa, M., Radomski, M., Carnuccio, R. and Moncada, S. (1991) Biochem. Biophys. Res. Commun. 172, 1246–1252.
[7] Perrella, M.A., Yoshizumi, M., Fen, Z., Tsai, J.-C., Hsieh, C.-M., Kourembanas, S. and Lee, M.-E. (1994) J. Biol. Chem. 270, 29541–29547.
[8] Green, L.C., Wagner, D.A., Glogowski, J., Skipper, P.L., Wishnok, J.S. and Tannenbaum, S.R. (1982) Anal. Biochem. 126, 131–138.
[9] Seo, H.G., Tatsumi, H., Fujii, J., Nishikawa, A., Suzuki, K., Kangawa, K. and Taniguchi, N. (1994) J. Biochem. 115, 602–607.
[10] Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. 162, 156–159.
[11] Kaneto, H., Fujii, J., Seo, H.G., Suzuki, K., Matsumoto, T., Nakamura, M., Tatsumi, H., Yamasaki, Y., Kamada, T. and Taniguchi, N. (1995) Diabetes 44, 733–738.
[12] Kennedy, B.P., Chan, C.-C., Culp, S.A. and Cromlish, W.A. (1993) Biochem. Biophys. Res. Commun. 197, 494–500.
[13] Shibahara, S., Muller, R., Taguchi, H. and Yoshida, T. (1985) Proc. Natl. Acad. Sci. USA 82, 7865–7869.
[14] Xie, Q.-W., Whisnant, R. and Nathan, C. (1993) J. Exp. Med. 177, 1779–1784.
[15] Xie, Q.-W., Kashiwabara, Y. and Nathan, C. (1994) J. Biol. Chem. 269, 4705–4708.
[16] Martin, E., Nathan, C. and Xie, Q.-W. (1994) J. Exp. Med. 180, 977–984.
[17] Spink, J., Cohen, J. and Evans, T.J. (1995) J. Biol. Chem. 270, 29541–29547.
[18] Abraham, N.G., Lin, J.H.-C., Schwartzman, M.L., Levere, R.D. and Shibahara, S. (1988) Int. J. Biochem. 20, 543–558.