

## DIFFERENT ACTION OF IBMX, ISOPROTERENOL AND RUTIN ON ORTHOVANADATE-INDUCED NITRIC OXIDE RELEASE IN MOUSE MACROPHAGE CELLS

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The effects of cAMP-elevating compounds IBMX (3-isobutyl-1-methylxanthine) and isoproterenol, and that of rutin (an effective superoxide scavenger) were studied on orthovanadate- (a putative protein-phosphotyrosine phosphatase inhibitor) induced nitric oxide (NO) production in J774A.1 mouse macrophage cells. As we previously reported (Koncz and Horváth, 2000), rutin and sodium orthovanadate act synergistically to induce production of high amount of NO in J774A.1 cells. IBMX, an agent that can elevate cAMP level in the cells, can reduce the production of both the LPS- and rutin + orthovanadate-induced NO in macrophages. In contrast, isoproterenol, a non-selective  $\beta$ -adrenergic receptor agonist, that reduced the LPS-induced NO production in macrophage cells, was unable to reduce the rutin + orthovanadate-induced NO production without negatively affecting cell viability. Moreover, isoproterenol dramatically enhanced the orthovanadate-induced NO synthesis in J774A.1 cells. Our previous study clarified that rutin and orthovanadate, in a specific concentration ratio of both, were able to produce hydrogen peroxide ( $H_2O_2$ ). Using 2',7'-dichlorofluorescein diacetate as a marker for  $H_2O_2$ , isoproterenol alone induced its oxidation but the rutin plus orthovanadate-induced  $H_2O_2$  production was reduced by isoproterenol. These observations have revealed that, in some cases,  $H_2O_2$  and superoxide ( $O_2^-$ ) scavengers can act in a reverse mode on macrophage cells depending on the presence or absence of orthovanadate.

**Key words:** cAMP, sodium orthovanadate, rutin, hydrogen peroxide, nitric oxide, J774A.1 cell line

*List of abbreviations:* **cAMP:** cyclic-adenosine-3',5'-monophosphate; **DCFH-DAC:** 2',7'-dichlorofluorescein diacetate; **DMSO:** dimethyl sulphoxide; **FBS:** fetal bovine serum;  **$H_2O_2$ :** hydrogen peroxide; **IBMX:** 3-isobutyl-1-methylxanthine; **iNOS:** inducible nitric oxide synthase; **ISO:** Isoproterenol; **LAL Test:** Lymulus Amebocyte Lysate Test; **L-NOARG:**  $N^G$ -nitro-L-arginine; **LPS:** bacterial lipopolysaccharide; **MTT:** 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; **NF- $\kappa$ B:** nuclear factor- $\kappa$ B; **NO:** nitric oxide; **NOS:** nitric oxide synthase;  **$O_2^-$ :** superoxide anion; **PDTc:** pyrrolidine dithiocarbamate; **PMA:** phorbol-12-myristate-13-acetate; **SOD:** superoxide dismutase;  **$VO_4$ :** (sodium) orthovanadate

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Nitric oxide (NO) has been identified as an important signalling molecule and plays a key role in the defence mechanisms of the immune system and in the pathogenesis of some inflammatory and non-inflammatory diseases. NO is generated by the nitric oxide synthase (NOS) enzyme from L-arginine and molecular oxygen. Three isoforms of NOS have been identified to date. In macrophages, the  $\text{Ca}^{2+}$ /calmodulin independent inducible nitric oxide synthase (iNOS) is responsible for the production of high amounts of NO contributing to the cytotoxic properties of these cells against target cells (Hibbs et al., 1988). Also, nitric oxide can cause vasorelaxation via activation of soluble guanylyl cyclase in endothelial cells (Olson et al., 1997). The induction of NO by LPS in J774A.1 mouse macrophage cells has been inhibited by cAMP elevating agent IBMX (Pang and Houlst, 1997). Isoproterenol had the same inhibitory effect on NO synthesis by LPS-induced rat Kupffer's cells (Mustafa and Olson, 1998), rat astroglial cultures (Feinstein et al., 1993) and RAW-267.7 mouse macrophage cells (Hasko et al., 1998), as well. In contrast to this, isoproterenol significantly increased nitrite production by IL-1 beta-stimulated rat cardiac myocytes (Kurosaki et al., 2000). On the contrary, NO decreased the cardiac myocyte contractile function of isoproterenol (Ungureanu-Longrois et al., 1995) and the potency of ISO via oxidation of it (Klatt et al., 2000).

Isoproterenol is a non-selective  $\beta$ -adrenergic receptor agonist.  $\beta$ -adrenergic receptor agonists are known immunosuppressant agents, and ISO is a known  $\text{H}_2\text{O}_2$  and  $\text{O}_2^-$  scavenger (Goeptar et al., 1988; Gillissen et al., 1997; Diez-Fraile et al., 2000). Isoproterenol is a potential effective vasodilator in the treatment of chronic obstructive pulmonary disease in horses (Pearson and Riebold, 1989) and it is a potential effective agent for treatment of inherited lethal ventricular arrhythmias in dogs (Sosunov et al., 2000). Moreover, it can cause lipolysis in underfed ewes via stimulation of beta-adrenergic receptors on adipocytes (Ferlay et al., 2001). ISO also stimulates glucose oxidation in rat hearts (Zimmer et al., 1990; Olley et al., 1996).

Although ISO is widely used in the treatment of bronchial asthma (Walters and Walters, 2000), it can cause severe impairments in humans and animals as well (Beasley et al., 1999; Hirono et al., 2001).

First of all, myocardial ischaemia and infarction is caused by administration of ISO (Blasig et al., 1985; Rona, 1985; Mathew et al., 1986). The resulting oxidative injury of cells could be the main cause of these negative effects of ISO (Janatova et al., 1986; Persoon-Rothert et al., 1989). ISO is able to go through auto-oxidation and induces several cell types consequently (Rosa, 1997; Rathore et al., 1998; Rathore et al., 2000; Remiao et al., 2000; Tappia et al., 2001).

Several effects of isoproterenol on  $\beta$ -adrenergic receptor bearing cells have been antagonised by sodium orthovanadate mainly due to its protein-tyrosine phosphatase inhibitory properties (Hudgins and Bond, 1979; Nieder et al., 1979; Churchill and Churchill, 1982; D'Ocon, 1989; Gordon, 1991; Sims et

al., 2000). Due to the insulin-like effects of  $\text{VO}_4$ , it can antagonise the effects of ISO on lipolysis (Mooney et al., 1989; Li et al., 1997; Castan et al., 1999) as well.

According to our previous study (Koncz and Horváth, 2000), orthovanadate can induce NO production of J774A.1 mouse macrophage cells acting synergistically with rutin. Orthovanadate can also potentiate PMA-induced superoxide generation in rat alveolar macrophages upon prolonged incubation (Mayer et al., 1995), stimulate the MAP kinase pathway and phosphatidylinositol 3-kinase (Pandey et al., 1998). Orthovanadate has been previously reported to induce expression of several genes in mouse C127 cells: actin, c-Ha-ras and c-jun (Yin et al., 1992). Orthovanadate can also potentiate IL-1 beta-induced NO release to the medium from the insulin-producing cell line RINm-5F via inhibition of protein tyrosine phosphatases (Welsh, 1994), and caused synthesis of endothelium-derived NO in pigs via endothelial tyrosine kinases and pertussis toxin-sensitive G-proteins (Nakaike et al., 1996). Because of its insulin-like properties,  $\text{VO}_4$  was effective in the treatment of diabetes in rats (Ozcelikay et al., 1993).

Several vanadium compounds are in our environment as pollutants (Hansard et al., 1982; Barceloux, 1999). Vanadate in some cases can cause asthma (Cortijo et al., 1997). Orthovanadate is capable of causing oxidative burst in macrophages (Green et al., 1992; Green and Phillips, 1994; Bassal et al., 1997). Vanadyl hydroperoxide enhances phosphorylation more than vanadate (Dackiw et al., 1997). Orthovanadate plus  $\text{H}_2\text{O}_2$  induce several genes in THP1 macrophages via oxidative stress (Mietus-Snyder et al., 1998).

Among flavonoids, there are several compounds with effective NO scavenging activity (van Acker et al., 1995). Quercetin and its 3-rutinoside derivative, rutin, are known cytoprotective agents against oxidative injury (Jenkins et al., 1992; Thompson et al., 1999).

As both rutin and quercetin were able to augment the NO production of orthovanadate-induced J774A.1 macrophage cells (Koncz and Horváth, 2000), cAMP elevating agents (i.e. ISO and IBMX; Safrany and Shears, 1998) have been tested on orthovanadate-induced NO production in J774A.1 cells. As macrophages bear  $\beta_2$ -adrenergic receptors (Izeboud et al., 2000), and isoproterenol is a potent superoxide anion and hydrogen peroxide scavenging agent, we expected the inhibition of NO production of  $\text{VO}_4$  or rutin +  $\text{VO}_4$  induced J774A.1 cells.

## Materials and methods

### *Materials*

DCFH-DAC was purchased from Molecular Probes Inc. (Eugene, Oregon, USA). LAL Test was purchased from Whittaker Bioproducts (Walkersville, MD, USA). Catalase [E.C. 1.11.1.6.] and hydrogen peroxide (30%) were purchased

from Reanal Fine Chemicals Co. (Budapest, Hungary). All other reagents and test compounds were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

#### *Effects on NO production and viability of J774A.1 cells*

J774A.1 mouse macrophage cells (ATCC TIB-67, Rockville, MD, USA) were maintained according to our previous experiments (Koncz and Horváth, 2000). Briefly, cells were propagated in RPMI-1640 (90%), FBS (10%) medium (McCall et al., 1991). The potential LPS contamination of the medium during the maintenance of cells was checked by the LAL Test. For experiments, J774A.1 cells were thoroughly washed with FBS-free RPMI-1640 medium and seeded out at the concentration of  $10^5$  cells/well in 96-well plates in RPMI-1640 medium (without phenol red, named as medium in the text below).

Test compounds rutin, quercetin, isoproterenol, IBMX, genistein, cycloheximide, tyrphostin-AG126 and PDTC were dissolved (0.1 M) in DMSO, and were further diluted by medium. Sodium orthovanadate was dissolved directly in medium. LPS (*E. coli*, 055:B5) was diluted directly in FBS from a stock solution of 200 µg/ml, and it was used at the optimal concentration of 100 ng/ml on the cells.

The total volume of medium was 100 µl/well. After a 24-h incubation period, the nitrite content of the supernatants (triplicates in all experiments) was measured by the Griess reaction adapted for a 96-well plate reader (Green et al., 1982). Briefly, 50 µl of Griess reagent (1% w/v sulphanilamide and 0.1% w/v naphthylethylenediamine in 5% v/v phosphoric acid) was added to 50 µl of sample culture medium, and the plates were read after a 10-min incubation time, at room temperature (Elx800 ELISA Reader, Bio-Tek Instruments, Inc., Winooski, VT, USA). In all experiments, at the final concentration of 1 mM L-NOARG was used as control (Gross et al., 1990).

ANOVA and Newman-Keuls test were used to determine the significance between the means. The differences were taken as significant in case of  $P < 0.05$ .

After the experiments, the viability of J774A.1 cells was checked by MTT using the method of Schmitt et al. (1995), and measured at 550/690 nm by Elx800 ELISA Reader. The viability of untreated cells was taken as 100%.

#### *Effects on DCFH-DAC oxidation*

The effect of rutin, isoproterenol and sodium orthovanadate on the oxidation of DCFH-DAC was measured by the method of Roy et al. (1995) with a little modification. Briefly, the J774A.1 cells ( $10^6$ /ml in 1.5 ml, 0.01 M PBS, pH = 7.4) were incubated at 37 °C with DCFH-DAC (at the final concentration of 10 µM) for 2 min. The changes in fluorescence were measured at  $E_x = 503$  nm and  $E_m = 520$  nm after adding the test compounds, using a computer-controlled SHIMADZU-RF2001 spectrofluorimeter.

## Results

### *Effects of cAMP elevating agents on LPS-induced NO production*

At the concentrations used, neither the compounds (LPS, L-NOARG, rutin, quercetin,  $\text{VO}_4$ , IBMX, ISO, genistein, cycloheximide, tyrphostin-AG126, PDTC) nor catalase affected the Griess reaction.

The lack of FBS in the medium of J774A.1 cells caused no nitric oxide production of LPS- (100 ng/ml) treated cells (data not shown). Therefore, the potential LPS contamination of the compounds could not induce NO synthesis of the cells.

cAMP-elevating agents IBMX and isoproterenol were tested with LPS (Fig. 1A) or sodium orthovanadate + rutin. As it is shown in Fig. 1A, not only IBMX and isoproterenol but also rutin is able to reduce the level of nitric oxide synthesised by 100 ng/ml LPS induced J774A.1 cells. Only IBMX has reduced the viability of macrophage cells significantly at its highest concentration ( $10^{-3}$  M, Fig. 1B).

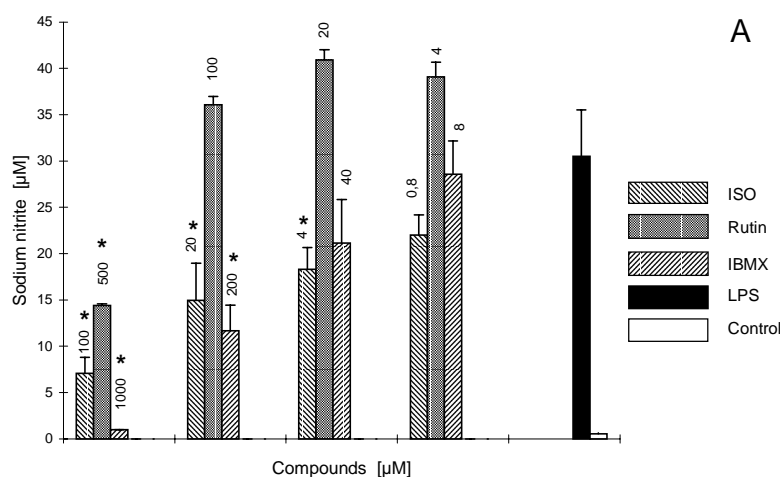


Fig. 1A. Effect of isoproterenol (ISO), IBMX and rutin on the nitric oxide production of bacterial lipopolysaccharide- (LPS, 100 ng/ml) induced J774A.1 cells. The concentration of ISO, IBMX and rutin is shown on the top of the columns ( $\mu\text{M}$ ). \*The differences between the mean values were significant ( $P < 0.05$ ) as compared to the value of LPS

### *Effects of cAMP-elevating agents on rutin + orthovanadate-induced NO production*

The most effective doses were 50  $\mu\text{M}$  for orthovanadate and 100  $\mu\text{M}$  for rutin. The absolute values of the measured nitrite concentrations in the supernatants of the cells varied between 7 and 40  $\mu\text{M}$ , and were always significantly higher than that of the control values (0–1.5  $\mu\text{M}$ ). L-NOARG, used at the concentration of 1 mM, always lowered the nitrite content of the supernatants of the cells by about 90–100%, without affecting the viability of cells negatively (data not shown).

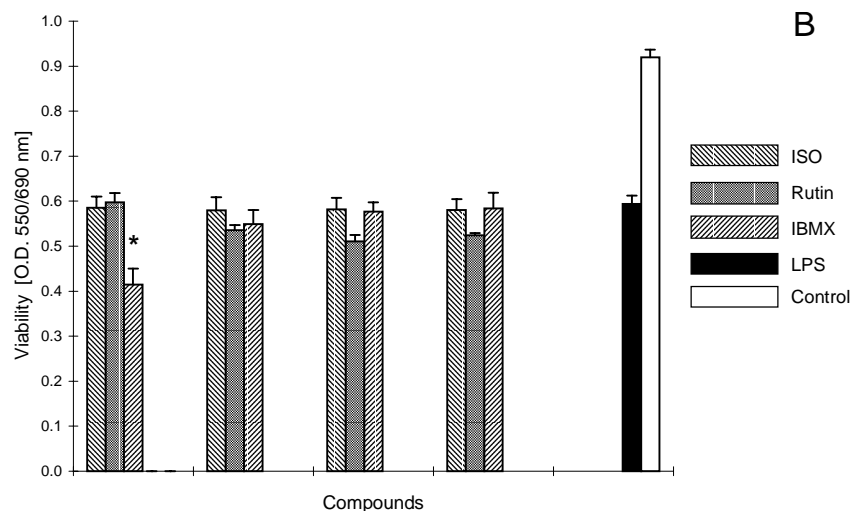


Fig. 1B. The effect of ISO, IBMX and rutin on the viability of the cells measured by MTT. The mean values of triplicates  $\pm$  standard deviations (S.D.) shown are representative of at least three experiments with similar results. \*The differences between the mean values were significant ( $P < 0.05$ ) as compared to the value of LPS

In the case of rutin + orthovanadate-induced NO synthesis in J774A.1 cells, IBMX was able to reduce the synthesised NO level without negatively affecting cell viability (Fig. 2B).

IBMX alone or in combination with rutin or orthovanadate was unable to induce NO synthesis (Fig. 2A).

Isoproterenol also decreased the rutin +  $\text{VO}_4$ -induced NO synthesis by macrophage cells, but it had significant negative effects on cell viability (Figs 3A and 3B). The incubation of cells with isoproterenol + rutin led to a slight increase of synthesised NO. In contrast, isoproterenol +  $\text{VO}_4$  were able to induce a marked level of NO from cells in a concentration-dependent manner. At 50  $\mu\text{M}$  of isoproterenol, there was a peak in the NO level synthesised by J774A.1 cells (Fig. 3A). Like in the case of incubation of isoproterenol with rutin + orthovanadate, the cell viability was affected negatively in the latter case as well. According to our previous results, similar effects have been observed with hydrogen peroxide + orthovanadate (Koncz and Horváth, 2000). Therefore, it is uncertain whether the inhibitory effect of isoproterenol on rutin + orthovanadate-induced NO synthesis was due only to the cAMP-elevating effect of isoproterenol.

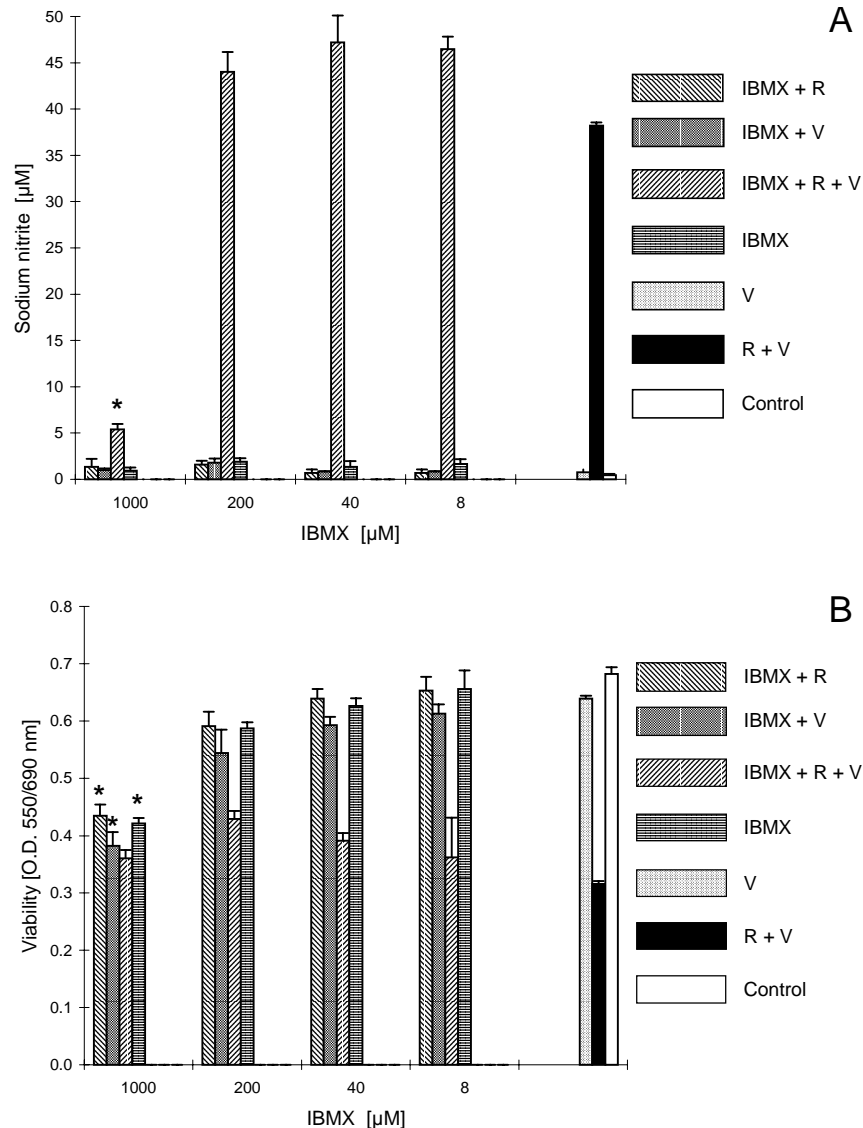


Fig. 2. Effect of different concentrations of IBMX on rutin (R, 100  $\mu\text{M}$ ) + orthovanadate- (V, 50  $\mu\text{M}$ ) induced NO synthesis (**A**) and viability (**B**) of J774A.1 cells. The means  $\pm$  S.D. values of triplicates shown are representative of three experiments with similar results.

\*The differences between the mean values were significant ( $P < 0.05$ ) as compared to the value of rutin + orthovanadate (R+V, **A**) or that of the control (**B**)

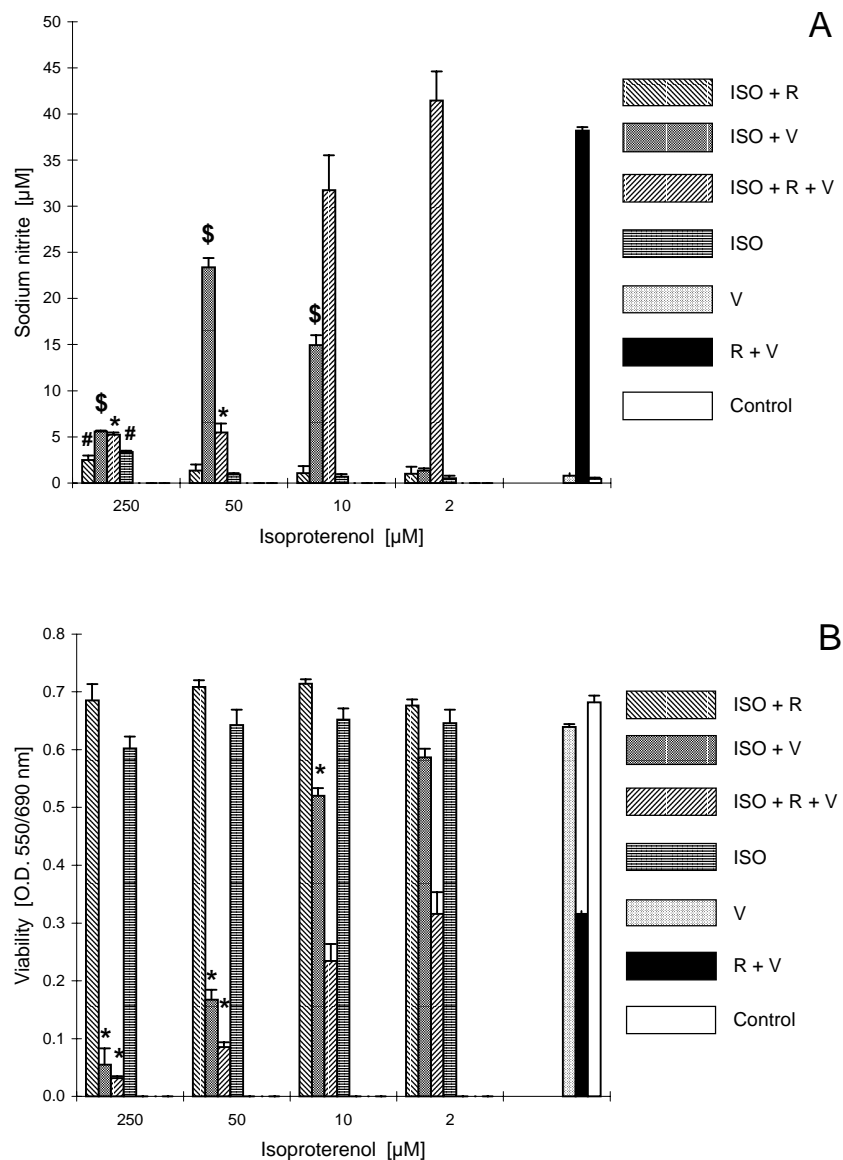


Fig. 3. Effect of different concentrations of isoproterenol (ISO) on rutin (R, 100  $\mu\text{M}$ ) + orthovanadate- (V, 50  $\mu\text{M}$ ) induced NO synthesis (A) and viability (B) of J774A.1 cells. The means  $\pm$  S.D. values of triplicates shown are representative of three experiments with similar results. \*The differences between the mean values were significant ( $P < 0.05$ ) as compared to the value of rutin + orthovanadate (R+V, A) or that of the control (B). <sup>§</sup>The differences between the mean values were significant ( $P < 0.05$ ) as compared to the value of orthovanadate (V, A). <sup>#</sup>The differences between the mean values were significant ( $P < 0.05$ ) as compared to the value of control (A)



To clarify it, the effect of isoproterenol and hydrogen peroxide on NO synthesis and cell viability was investigated further. As it is shown in Fig. 4A, when rutin + orthovanadate induced less NO synthesis in macrophage cells, isoproterenol and also  $H_2O_2$  were able to further increase the production of NO from these cells. The maximum effects were reached at 10  $\mu M$  and 20  $\mu M$  for isoproterenol and  $H_2O_2$ , respectively. Above these concentrations, the amount of synthesised NO decreased due to the loss of cell viability (Fig. 4B).

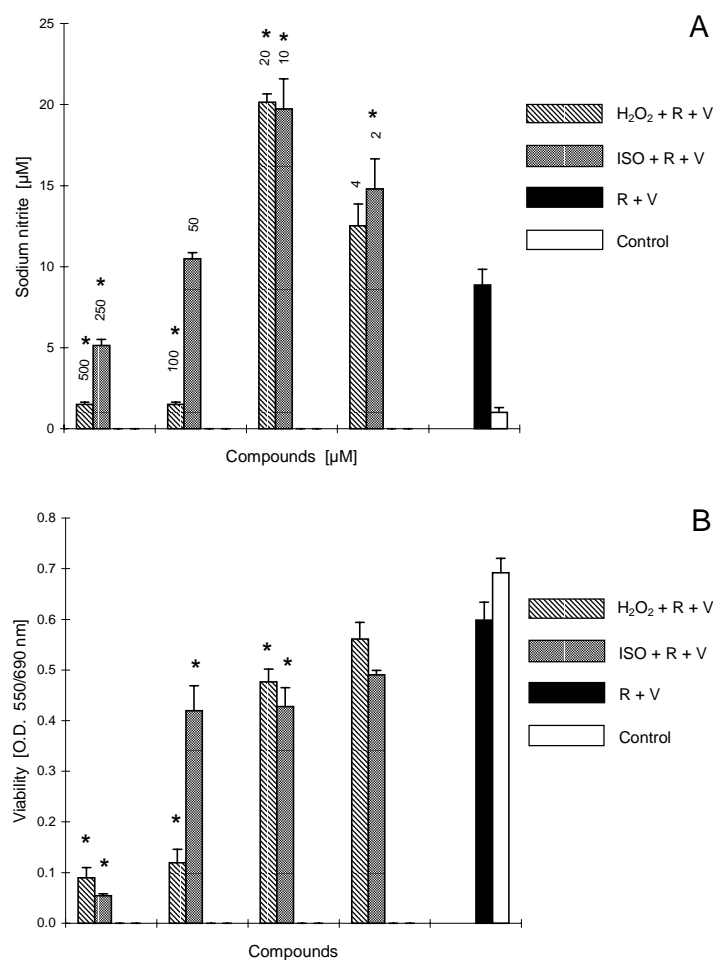


Fig. 4. Effect of hydrogen peroxide ( $H_2O_2$ ) and isoproterenol (ISO) on rutin (R, 100  $\mu M$ ) + orthovanadate- (V, 50  $\mu M$ ) induced NO synthesis (A) and viability (B) of J774A.1 cells. The concentrations of  $H_2O_2$  and ISO are shown on the top of the columns ( $\mu M$ ). Means of triplicates  $\pm$  S.D. values of a representative experiment are shown. Similar results were obtained in at least three independent experiments. \*The differences between the mean values were significant ( $P < 0.05$ ) as compared to the value of rutin + orthovanadate (R+V, A) or that of the control (B)

Therefore, it could be suggested that lowering of the synthesised NO level from rutin + orthovanadate-induced J774A.1 macrophage cells by isoproterenol is due to its negative effect on cell viability rather than to the cAMP-elevating effect of isoproterenol.

*Effects of specific inhibitors and scavengers on isoproterenol + orthovanadate-induced NO production*

To clarify the putative effects of NF- $\kappa$ B activation and that of tyrosine phosphorylation in the pathway leading to NO production, PDTC (NF- $\kappa$ B inhibitor), tyrphostin-AG126 and genistein (protein tyrosine kinase inhibitors) were used as test compounds. To check whether newly synthesised iNOS was responsible for the elevated amount of NO produced by induced macrophages, cycloheximide (translation inhibitor) was used.

Catalase ( $\text{H}_2\text{O}_2$  scavenger) and SOD ( $\text{O}_2^-$  scavenger, EC 1.15.1.1.) were used to clarify the putative role of reactive oxygen species (i.e.  $\text{H}_2\text{O}_2$  and  $\text{O}_2^-$ ) in the activation of macrophages.

The above-mentioned compounds and enzymes were tested, giving them together at suboptimal concentrations of isoproterenol (2  $\mu\text{M}$ ) and orthovanadate (50  $\mu\text{M}$ ) to the cells. The hydrogen peroxide scavenger catalase could inhibit the effect of isoproterenol + orthovanadate without affecting the cell viability negatively (Table 1). Cycloheximide, PDTC, genistein and tyrphostin-AG126 also inhibited the NO synthesis induced by isoproterenol and orthovanadate without affecting the cell viability negatively (Table 1). SOD had no inhibitory effect on the action of isoproterenol + orthovanadate at the concentrations used (4–500 U/ml; data not shown).

**Table 1**

Effects of genistein, tyrphostin, catalase, PDTC and cycloheximide on the isoproterenol (ISO, 2  $\mu\text{M}$ ) plus orthovanadate- (V, 50  $\mu\text{M}$ ) or hydrogen peroxide- ( $\text{H}_2\text{O}_2$ , 20  $\mu\text{M}$ ) plus orthovanadate- (V, 50  $\mu\text{M}$ ) induced NO production and viability of J774A.1 cells. Means of triplicates  $\pm$  S.D. values are shown from a representative experiment. Similar results were obtained in at least three independent experiments. \*The differences between the mean values were significant ( $P < 0.05$ ) as compared to the value of (ISO + V) or that of the ( $\text{H}_2\text{O}_2$  + V)

Inhibitors and scavengers	ISO + V	$\text{H}_2\text{O}_2$ + V	ISO + V	$\text{H}_2\text{O}_2$ + V
	Sodium nitrite [ $\mu\text{M}$ ]		Viability [O.D. 550/690 nm]	
Genistein (100 $\mu\text{M}$ )	1.411 $\pm$ 0.202*	1.143 $\pm$ 0.116*	1.130 $\pm$ 0.030	1.103 $\pm$ 0.077
Tyrphostin (100 $\mu\text{M}$ )	1.680 $\pm$ 0.116*	1.344 $\pm$ 0.116*	0.931 $\pm$ 0.035	0.906 $\pm$ 0.023
Catalase (11.424 ng/ml)	0.604 $\pm$ 0.000*	0.605 $\pm$ 0.201*	0.887 $\pm$ 0.010	0.934 $\pm$ 0.014
PDTC (20 $\mu\text{M}$ )	0.874 $\pm$ 0.116*	0.807 $\pm$ 0.000*	0.918 $\pm$ 0.036	0.877 $\pm$ 0.023
Cycloheximide (0.2 mM)	0.739 $\pm$ 0.116*	0.672 $\pm$ 0.308*	0.848 $\pm$ 0.078	0.893 $\pm$ 0.062
Control	0.807 $\pm$ 0.202*	0.807 $\pm$ 0.202*	1.039 $\pm$ 0.043	1.039 $\pm$ 0.043
(ISO + V) or ( $\text{H}_2\text{O}_2$ + V)	8.523 $\pm$ 0.620	4.570 $\pm$ 0.953	0.894 $\pm$ 0.047	0.878 $\pm$ 0.007

Therefore, we suggest that the action of isoproterenol with orthovanadate was mediated, at least partially, via liberation of  $\text{H}_2\text{O}_2$ .

#### *Effects on DCFH-DAC oxidation*

To clarify the putative role of isoproterenol in the liberation of  $\text{H}_2\text{O}_2$  during its incubation with orthovanadate, we used DCFH-DAC as hydrogen peroxide indicator. In the reaction mixture, the concentration of DMSO was 1%, and it did not affect the oxidation of DCFH-DAC (data not shown). The studies on the effects on DCFH-DAC oxidation revealed that DCFH-DAC was mildly oxidised by isoproterenol alone. Adding orthovanadate to the mix of isoproterenol and DCFH-DAC, a decrease in  $\text{H}_2\text{O}_2$  liberation was observed (Fig. 5).

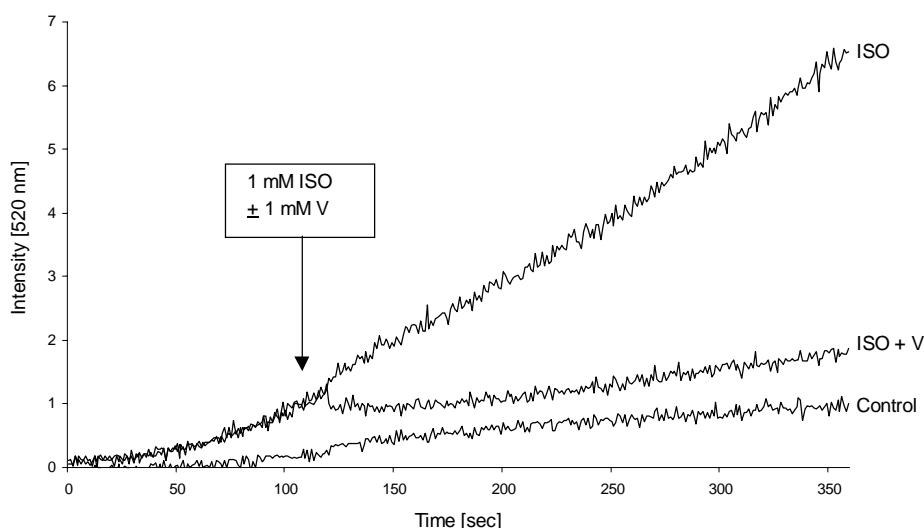


Fig. 5. Effects of isoproterenol (ISO, 1 mM) and orthovanadate (V, 1 mM) on the oxidation of DCFH-DAC. J774A.1 cells ( $10^6$  cells/ml) had been incubated with  $10 \mu\text{M}$  DCFH-DAC in 0.01 M PBS for 2 min before adding the drugs at the concentrations indicated. The oxidation was followed at  $E_x = 503 \text{ nm}$  and  $E_m = 520 \text{ nm}$ . Data from a single experiment are shown. Similar results were obtained in at least two independent experiments

When isoproterenol was used in combination with rutin + orthovanadate during DCFH-DAC oxidation, it was revealed that the higher concentration of isoproterenol resulted in a lower concentration of liberated  $\text{H}_2\text{O}_2$  (Fig. 6).

In contrast, incubating orthovanadate with various concentrations of rutin, higher concentrations of rutin resulted in higher concentrations of liberated  $\text{H}_2\text{O}_2$  (Fig. 7).

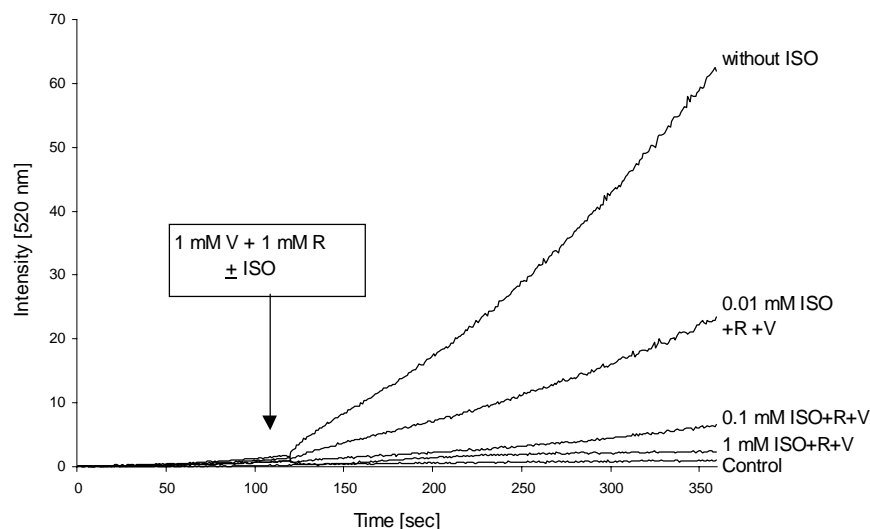


Fig. 6. Effects of different concentrations of isoproterenol (ISO) on rutin (R, 1 mM) plus orthovanadate- (V, 1 mM) induced oxidation of DCFH-DAC. J774A.1 cells ( $10^6$  cells/ml) had been incubated with  $10 \mu\text{M}$  DCFH-DAC in 0.01 M PBS for 2 min before adding the drugs at the concentrations indicated. The oxidation was followed at  $E_x = 503 \text{ nm}$  and  $E_m = 520 \text{ nm}$ . Data from a single experiment are shown. Similar results were obtained in at least two independent experiments

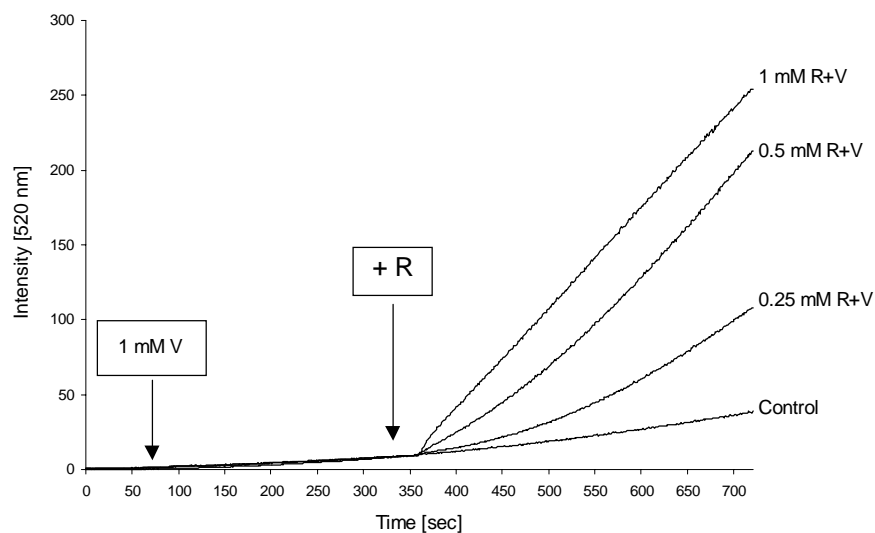


Fig. 7. Effects of different concentrations of rutin (R) plus orthovanadate (V, 1 mM) on oxidation of DCFH-DAC. J774A.1 cells ( $10^6$  cells/ml) had been incubated with  $10 \mu\text{M}$  DCFH-DAC in 0.01 M PBS for 2 min before adding the drugs at the concentrations indicated. The oxidation was followed at  $E_x = 503 \text{ nm}$  and  $E_m = 520 \text{ nm}$ . Data from a single experiment are shown. Similar results were obtained in at least two independent experiments

### Discussion

The action of cAMP-elevating agents IBMX and isoproterenol on LPS-, orthovanadate- and orthovanadate + rutin-induced nitric oxide synthesis of J774A.1 mouse macrophage cells has been investigated. Both test compounds were able to reduce LPS-induced NO production of the cells. Rutin, as an effective oxygen radical scavenger, also inhibited NO production in the cells induced by LPS.

As regards rutin + VO<sub>4</sub>-induced NO production, only IBMX inhibited it without affecting cell viability negatively.

In cases when cells produced a lower amount of NO on induction by orthovanadate + rutin, both isoproterenol and H<sub>2</sub>O<sub>2</sub> were able to further increase the NO production of the macrophages. This effect of ISO might be attributed to its capability of auto-oxidation and not to the augmentation of intracellular cAMP level. These observations suggested the putative role of H<sub>2</sub>O<sub>2</sub> during the action of isoproterenol on the cells.

Among the cAMP-elevating compounds tested only isoproterenol was able to augment the synthesis of NO in synergy with orthovanadate. Orthovanadate alone caused no significant NO production of the cells.

The inhibitory action of cycloheximide, a protein synthesis inhibitor (Obrig et al., 1971) and that of L-NOARG (Gross et al., 1990) means that the inducible form of nitric oxide synthase (iNOS) was synthesised in the macrophage cells during activation.

SOD (Flohé and Ötting, 1984) was unable to inhibit the action of ISO + orthovanadate on J774A.1 cells. In contrast, catalase, a hydrogen peroxide scavenging enzyme (Aebi, 1984), could inhibit the production of NO, parallel with increasing cell viability. Because of the fact that DCFH-DAC is not sensitive to the superoxide anion (Lebel et al., 1992; Zhu et al., 1994), the role of superoxide anion in the action of ISO + orthovanadate could be completely excluded.

These results suggest that the first step of induction and production of NO by ISO + orthovanadate was related to the production of hydrogen peroxide at the external surface of the cells. The scheme of the signal transduction activating and inactivating pathways leading to NO synthesis in macrophages is shown in Fig. 8.

The effect of ISO on DCFH-DAC oxidation revealed that a low amount of H<sub>2</sub>O<sub>2</sub> might be generated during its auto-oxidation. As ISO inhibited the rutin + orthovanadate-induced DCFH-DAC oxidation by scavenging hydrogen peroxide, this further verified our previous hypothesis, i.e. that H<sub>2</sub>O<sub>2</sub> plays a key role in the induction of NO by rutin + VO<sub>4</sub> in relation to the induced macrophage cells.

The fact that ISO was unable to cause DCFH-DAC oxidation together with orthovanadate might mean that auto-oxidation of ISO was in competition with its scavenging function. As catalase was able to inhibit ISO + VO<sub>4</sub>-induced NO production of J774A.1 cells, the final role of hydrogen peroxide in induction of these cells seems to be undoubted.

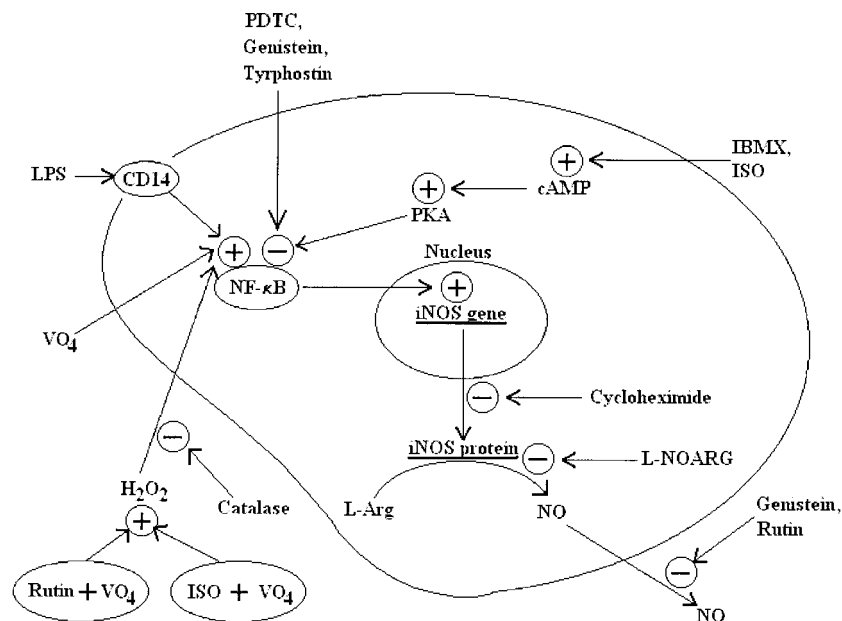


Fig. 8. Schematic illustration of the effects of compounds on NO production of a macrophage cell. +: increase or activation, -: decrease or inactivation; →: direct or indirect effect of the specified compound. Further abbreviations are found in the appendix

Hydrogen peroxide can react with orthovanadate, forming peroxovanadate that can activate the cells thereafter (Kazazi et al., 1996). Among the targets of these oxidative agents, there is NF-κB (Meyer et al., 1993). Although pervanadate inhibits the I-κB degradation and NF-κB translocation to the nucleus in TNF-α treated cells (Menon et al., 1995; Singh and Aggarwal, 1995), pervanadate alone can activate NF-κB by an alternative pathway, without proteolytic degradation of I-κB (Imbert et al., 1996). After activation, NF-κB can induce the transcription of several genes, for example that of iNOS in macrophages (Schreck et al., 1992; Sherman et al., 1993).

The role of NF-κB in mediating the action of rutin + orthovanadate is supported by the fact that PDTC could inhibit the NO production of cells activated by ISO + orthovanadate without affecting cell viability negatively.

The inhibitors of protein tyrosine kinases were able to block ISO + orthovanadate-induced NO synthesis. As genistein also has a hydrogen peroxide scavenging activity (Akiyama et al., 1987; Wei et al., 1993; Wei et al., 1995), its effective inhibitory dose was lower than that of tyrphostin-AG126 (Novogrodsky et al., 1994). Since protein tyrosine kinase inhibitors can directly inhibit the activation of NF-κB (Singh and Aggarwal, 1995), the activation of NF-κB by hydrogen peroxide and orthovanadate and/or peroxovanadate can be prevented by genistein and tyrphostin-AG126.

The first step in the action of ISO plus orthovanadate toward the induction of iNOS in J774A.1 macrophages might be the vanadate-catalysed auto-oxidation of ISO, followed by the activation of NF- $\kappa$ B.

In the literature, several articles deal with the joint action of isoproterenol and orthovanadate on several cell types. In the light of our recent results, we strongly suggest the consideration of the potential oxidative side effects of these two compounds (i.e. generation of H<sub>2</sub>O<sub>2</sub>) and, if it is possible, the prevention of auto-oxidation of ISO by catalase.

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