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SPECIAL REPORT

Enhancing effect of staurosporine on NO production in rat peritoneal macrophages via a protein kinase C-independent mechanism

Grzegorz Sowa & [1]Ryszard Przewłocki

Neuropeptide Research Department, Institute of Pharmacology, Polish Academy of Sciences, 31-343 Kraków, 12 Smętna Street, Poland

Staurosporine (3-100 nM), frequently used as a protein kinase C (PKC) inhibitor, increased accumulation of nitrite in the culture medium of rat peritoneal macrophages up to 6 times above the control level. Moreover, when used in combination with the stable analogue of cyclic AMP, dibutyryl-cyclic AMP (db cyclic AMP; 0.1 mM), and/or a cytokine, tumour necrosis factor-α (TNFα; 100 u ml^{-1}), staurosporine synergistically potentiated, up to 30 times, nitrite accumulation. On the other hand, the other PKC inhibitors, calphostin C and H-7 (10 nM-10 μM) were not effective under the same conditions. The staurosporine-induced nitrite accumulation, in both the presence and the absence of TNFα and/or db cyclic AMP was effectively inhibited by the protein synthesis inhibitor, cycloheximide, or by the nitric oxide (NO) synthesis inhibitor, N^G-monomethyl-L-arginine (L-NMMA). Thus our data suggest that staurosporine may enhance NO production in macrophages via intracellular mechanisms unrelated to the PKC inhibition.

Keywords: Staurosporine; nitric oxide (NO); cyclic AMP; tumor necrosis factor-α (TNFα); (rat) peritoneal macrophages

Introduction Production of nitric oxide (NO) in activated macrophages, which is an important cytotoxic/cytostatic mechanism of nonspecific immunity, has been very well documented (see review by Moncada *et al.*, 1991). This process is initiated by 'induction' of the inducible NO synthase (iNOS), involved in the synthesis of both mRNA for the protein and the protein itself. The induction of iNOS and NO synthesis in macrophages may be caused by activators of these cells, including such bacterial products as lipopolysaccharide, muramyl dipeptide (Moncada *et al.*, 1991), some exotoxins, e.g. cholera toxin (Sowa & Przewłocki, 1994) and host cell products, especially cytokines such as interferon-γ, tumour necrosis factor-α (TNFα) and interleukin-1 (Moncada *et al.*, 1991). However, little is known about induction of NO synthesis by factors which do not belong to these groups, in particular fungal products, since NO produced by macrophages may also act as a fungicidal agent (Moncada *et al.*, 1991). The present study shows that staurosporine, a fungal alkaloid produced by *Streptomyces staurosporus* (Omura *et al.*, 1977), commonly used as a protein kinase C (PKC) inhibitor (Tamaoki, 1991), can enhance NO production by rat peritoneal macrophages. Intracellular mechanisms of the staurosporine-induced enhancement of NO production seem to be PKC-independent and remain to be elucidated.

Methods Rat peritoneal macrophages were prepared and incubated for 44 h with the appropriate substances as described previously (Sowa & Przewłocki, 1994). The measurement of accumulated nitrites, stable endproducts of NO in *in vitro* systems, seems to be the best way of evaluating the level of NO synthesis by iNOS (Ignarro *et al.*, 1993). Accumulation of nitrites was measured with a Griess reagent (Sowa & Przewlocki, 1994) and calculated as nmol of nitrite per 10^5 cells 44 h^{-1} from four independent experiments. The statistical significance was evaluated by Student's unpaired *t* test. The EC_{50} values were calculated by a Graph Pad Programme (Graph Pad, San Diego, U.S.A.).

[1] Author for correspondence.

The following drugs were used: the PKC inhibitor staurosporine was obtained from two independent sources: Sigma Chemical Co. (St. Louis, MO, U.S.A.) and Calbiochem-Novabiochem (San Diego, CA, U.S.A.); the PKC inhibitors calphostin C and H-7 (1-(5-isoquinolinylsulphonyl)-2-methylpiperazine) came from Calbiochem-Novabiochem (San Diego, CA, U.S.A.); the cyclic AMP analogue dibutyryl-cyclic AMP (db cyclic AMP); a cytokine, TNFα; the reversible inhibitors of protein and NO synthesis cycloheximide and N^G-monomethyl-L-arginine (L-NMMA), respectively, were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Results The level of nitrite accumulated in the culture medium of unstimulated macrophages was usually relatively low but significant (about 0.27 ± 0.12 nmol per 10^5 cells 44 h^{-1}). Staurosporine (3-100 nM) alone significantly increased nitrite accumulation in a concentration-dependent manner 2.4, 3.8, 5.4 and 5.7 fold for concentrations of 3, 10, 30, and 100 nM, respectively ($EC_{50} \approx 6$ nM) (Figure 1). At higher concentrations, staurosporine caused no increase (not shown). db cyclic AMP (0.1 mM), which alone significantly increased accumulation up to 2 fold, tripled the effects of staurosporine at concentrations of 3 and 10 nM and doubled them at concentrations of 30 and 100 nM. The EC_{50} value for staurosporine in combination with db cyclic AMP was approximately 3 nM. TNFα (100 u ml^{-1}), which alone had only a slight but significant enhancing influence (about 2 fold), almost doubled the effects of staurosporine (1-100 nM). A combination of TNFα and db cyclic AMP synergistically increased 5 fold nitrite accumulation. Staurosporine potently ($EC_{50} \approx 5$ nM) and very effectively (more than 6 times at a relatively low concentration of 10 nM) potentiated the effect of the above combination. Nitrite accumulation caused by staurosporine alone or in combination with db cyclic AMP and/or TNFα was also time-dependent and lasted no more than 44 h (not shown). In contrast to staurosporine, calphostin C and H-7 (10 nM-10 μM) themselves did not induce increased nitrite accumulation nor did their presence affect nitrite accumulation in response to TNFα and/or db cyclic AMP (not shown). The stimulating effects of staurosporine (30 nM), alone or in combination with TNFα and/or db cyclic

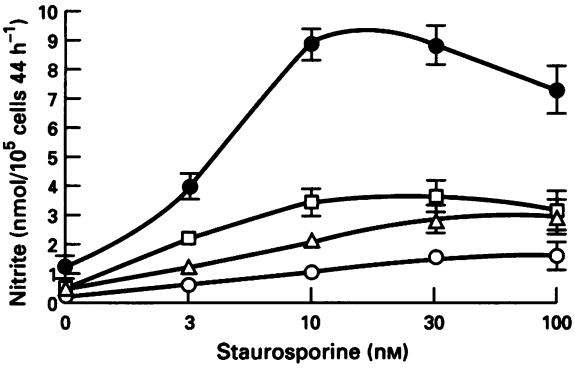

Figure 1 The potentiating effects of staurosporine (3–100 nM) on nitrite accumulation in the culture medium of rat peritoneal macrophages untreated (○), or treated with dibutyryl cyclic AMP (db cyclic AMP; 0.1 mM) (□), tumour necrosis factor-α (TNFα; 100 u ml^{-1}) (△), or both (●). The results are the mean ± s.e.mean from four independent experiments. All the points indicating the stimulating effects of staurosporine, db cyclic AMP, and TNFα alone are statistically significant ($P < 0.05$) vs an unstimulated control. In addition all points denoting the stimulating effects of staurosporine in combination with db cyclic AMP, TNFα, or with db cyclic AMP + TNFα are statistically significant vs the db cyclic AMP-, TNFα-, and db cyclic AMP + TNFα-induced stimulations, respectively, as well as vs those induced by the respective concentrations of staurosporine alone.

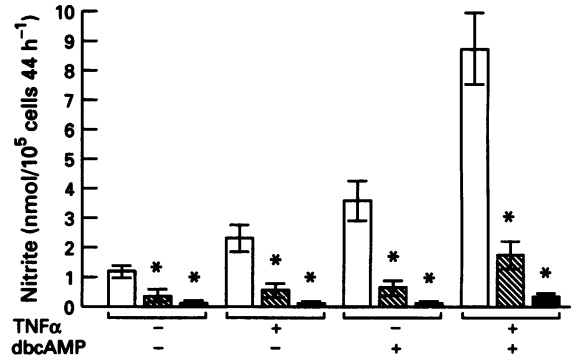

Figure 2 Stimulation of nitrite accumulation in the culture medium of rat peritoneal macrophages by staurosporine (30 nM) alone, or by its combination with dibutyryl cyclic AMP (db cyclic AMP; 0.1 mM) and/or with tumour necrosis factor-α (TNFα; 100 u ml^{-1}) (open columns) and inhibition of nitrite accumulation by N^G-monomethyl-L-arginine (0.5 mM) (hatched columns), or cycloheximide (1 μM) (solid columns). The results are the mean ± s.e.mean from four independent experiments. *$P < 0.05$, a statistically significant suppression of staurosporine- or its respective combination with db cyclic AMP- and/or TNFα-induced nitrite accumulation.

AMP, were significantly inhibited by L-NMMA (0.5 mM) (on an average, by ca. 80%) and cycloheximide (1 μM) (on an average, by ca. 95%) (Figure 2).

Discussion To our knowledge, the present study provides the first proof that the fungal alkaloid, staurosporine alone may induce NO production in macrophages. Moreover, this alkaloid is also able to act synergistically with a direct activator of the cyclic AMP pathway, db cyclic AMP which alone is able to induce NO synthesis in rat peritoneal macrophages (Sowa & Przewłocki, 1994), with a cytokine, TNFα, or with a combination of the two latter drugs to induce NO production. The effects of staurosporine seem to be very specific, as shown by its relatively low (nanomolar) effective concentrations, and do not appear to be related to contamination with lipopolysaccharide. Lipopolysaccharide does not act synergistically with TNFα (unpublished data) or db cyclic AMP (Sowa & Przewłocki, 1994) as does staurosporine. Moreover, the stimulating effects of staurosporine did not depend upon the origin (place of purchase) of the substance (see Methods). The potentiating effects of staurosporine on nitrite accumulation may be predicted to be related to *de novo* synthesis of iNOS and subsequent NO synthesis, as it was inhibited by cycloheximide and L-NMMA, reversible inhibitors of protein and NO synthesis, respectively. The lack of a stimulating effect of staurosporine at its higher concentration was due to its cytotoxic properties as tested by trypan blue exclusion (not shown). Although staurosporine is conventionally used as a PKC inhibitor (Tamaoki, 1991), its stimulating effects did not stem from the PKC inhibition, since calphostin C and H-7, the two other PKC inhibitors, used at a relatively wide range of concentrations were not effective in increasing nitrite accumulation (not shown).

In conclusion, staurosporine, a nonspecific PKC inhibitor, alone appears to enhance NO production in rat peritoneal macrophages and to potentiate it in response to the cyclic AMP pathway activation and the TNFα-mediated intracellular signal via some still unknown intracellular mechanisms unrelated to the PKC inhibition. The potentiating effects of this fungal alkaloid suggest that other staurosporine-like fungal products might also be effective in stimulating NO production.

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