Activation of both poly (ADP-ribose) polymerase (PARP) and inducible nitric oxide synthase (NOS-2) have been implicated in the pathogenesis of various forms of inflammation, therefore compounds which may simultaneously inhibit both pathways are of potential therapeutic interest. We tested the influence of potent inhibitor of PARP, 1, 5-isoquinolinediol (ISO), on NOS-2 induction in model of mouse macrophages (cell line J774.2) stimulated with lipopolysaccharide (1 µg/ml). Pretreatment with ISO (1-300 µM) resulted in dose-dependent inhibition of accumulation of NOS-2-derived nitrite in culture medium (IC₅₀ = 9,3 µM) as well as inhibition of NOS-2 protein induction in cultured J774.2 cells; ISO given 10 hours after LPS did not influence activity of NOS-2. Interestingly, another PARP inhibitor, 3-aminobenzamide (3-AB, 10-3000 µM), did not influence 24-hr nitrite accumulation in J774.2 cell culture, either administered 15 minutes prior to LPS or 10 hrs after LPS. Scavenging of reactive oxygen species by use of mixture of SOD and catalase (SOD/Cat, 100/300 - 1000/3000 U/ml) as well as cell permeable SOD-mimetic [Mn(III)TBAP, 1- 100 µM], did not influence NOS-2 induction in J774.2 cells. In summary, we identified 1, 5-isoquinoline as potent inhibitor of induction of NOS-2 in LPS-treated mouse macrophages. The exact mechanism of inhibitory action of this compound on NOS-2 induction requires further investigation.

Key words: 1,5-isoquinolinediol, PARP inhibitors, macrophages, inducible NOS, lipopolysaccharide (LPS)

INTRODUCTION

Poly (ADP-ribose) polymerase (PARP, EC 2.4.2.30) is a nuclear enzyme that upon binding to DNA strand breaks catalyses the transfer of the ADP-ribose moiety of NAD⁺ to various nuclear proteins including histones and transcription
factors, forming an (ADP-ribose)$_n$ chain with branches (1,2). Physiologically, poly (ADP-ribosyl)ation has been implicated in multiple processes including DNA repair and maintenance of genomic integrity as well as in cellular differentiation, regulation of cytoskeletal organization and expression of various proteins (3-5). However, excessive activation of PARP in response to inflammatory or oxidative insults, via NAD$^+$ and ATP depletion leads to mitochondrial injury, cellular energy failure and death (3,6-9). This energy-consuming poly (ADP-ribosyl)ation has been implicated in the pathogenesis of stroke, myocardial ischemia, diabetes-associated cardiovascular dysfunction, shock, arthritis, and various other forms of inflammation, therefore inhibition of PARP activity may prove useful for the therapy of these diseases (10-13). Interestingly, in certain conditions, even interruption of physiological PARP activity may be considered beneficial. For example, impairment of DNA repair by pharmacological inhibition of PARP would limit the ability of cancer cells to repair, and thereby enhance the effects of cancer radiotherapy (14,15). Thus, PARP inhibitors may represent important, versatile group of drugs (16-18).

Induction of nitric oxide synthase (NOS-2) plays important role in aggravation of inflammation and cancerogenesis (19-24). Interestingly, several lines of evidences from genetic and pharmacological studies suggest mutual positive interactions between PARP and NOS-2 pathways (6,25). Accordingly, 3-aminobenzamide (3-AB) (Fig. 1 A), a classical, relatively weak inhibitor of PARP has been shown to inhibit induction of NOS-2, although in millimolar concentrations (26). Importantly, there is no data concerning influence of newer, more potent PARP inhibitors on NOS-2 induction.

Various structures containing carboxamide group incorporated within lactam ring structure were reported as more selective and effective at inhibiting PARP than 3-AB (27,28). Of these, 1, 5-isoquinolinediol (ISO) (Fig. 1 B), has proved to be potent PARP inhibitor in in vitro and in vivo applications (28,29).

In our work we tested the influence of 1, 5-isoquinolinediol on NOS-2 induction in mouse macrophages (cell line J774.2) stimulated with lipopolysaccharide.

![Fig. 1. Structures of PARP inhibitors: 1,5-isoquinolinediol (A) and 3-aminobenzamide (B).](image-url)
MATERIALS AND METHODS

Mouse Macrophage Cell Line J774.2

The mouse macrophage cell line J774.2 was cultured in T75 flasks in DMEM that contained 10% foetal bovine serum supplemented with streptomycin (100 µg/ml), penicillin (100 U/ml) and fungizone (0,25 µg/ml). Flasks were kept at 37 °C in atmosphere of humidified air containing 5% CO₂. Then, the cells were seeded in 96-well plates and cultured in 200 µl of culture medium until reaching confluence (10⁵ cells per well). Additionally, in order to obtain sufficient amount of material for mRNA and protein assays, the cells were cultured in 6-well plates (10⁶ cells per well) in 2 ml of culture medium. Nitric oxide synthase (NOS-2) in macrophages was induced by lipopolysaccharide from Escherichia coli serotype 0127:B8 (LPS, 1 µg/ml).

Protocol of experiments

The PARP inhibitors (1, 5-isoquinolinediol and 3-aminobenzamide) were given in fresh culture medium either 15 minutes prior to, or 10 hours after LPS (1 µg/ml) stimulation.

Culture medium and cells were harvested 24 hours after LPS stimulation.

Nitrite concentration in Culture Medium

Nitrite accumulation in J774.2 cell culture supernatant was measured using the Griess method as described previously (30). Briefly, 100 µl of 1% sulphanilamide in 5% phosphoric acid, followed by 100 µl of 0,1% N-(1-naphtyl)-ethylene diamine in 5% phosphoric acid were added to 100 µl of culture medium. After 10 minutes of incubation at 23 °C the absorbance at 550 nm was read. Micromolar concentrations of nitrite were calculated from standard curve constructed with sodium nitrite as a reference compound.

NOS-2 immunoblotting

24 hours after LPS stimulation the cells were lysed in lysis buffer (1% Triton X-100, 0,1% SDS in PBS containing 1 mM PMSF, 100 µM leupeptin, 50 µM peptatin A). Protein concentrations of lysates were determined using Bradford method. Samples, containing equal amounts of total protein were mixed with gel loading buffer [50 mM Tris, 10% SDS, 10% glycerol, 10% 2-mercaptoethanol, 2mg/ml bromophenol blue] in a ratio 1:1 (v/v) and boiled (4 min). Then samples (30 µg of total protein per lane) were separated on 7,5% SDS-polyacrylamide gels (Mini Protean II, Bio-Rad, USA) using Laemmli buffer system and proteins were semi-dry transferred to nitrocelulose membranes (Bio-Rad, USA). Non-specific binding sites were blocked overnight in 4 °C with 5% non-fat dried milk and the membranes were then incubated 2 hrs in room temperature (RT) with rabbit polyclonal antibody to NOS-2 (1: 2000) (Transduction Laboratories, USA). Bands were detected with alkaline phosphatase-conjugated secondary antibody (1 hr in RT, 1: 5000, Sigma, USA) and developed with BCIP and NBT (Sigma, USA). Rainbow markers (Amersham, USA) were used for molecular weight determinations. Protein bands were scanned and analyzed with freeware Scion image (Scion Corporation, USA).

Cell respiration assay

Cell respiration, an indicator of cell viability, was assessed by mitochondrial-dependent reduction of MTT to formazan. Cells in 96-well plates were incubated in 37°C with MTT (0,2 mg/ml for 60 minutes). Then, culture medium was removed by aspiration and cells were solubilized
in DMSO (200 µL). The extent of reduction of MTT to formazan within cells was quantitated by measurement of absorbance at 550 nm.

**Statistics**

All values in the figures and text are expressed as mean ± s.e.of n observations. A one way analysis of variance (ANOVA) followed, if appropriate, by a Bonferroni’s test for multiple comparisons was used to compare means between the groups. A P value less than 0.05 was considered to be statistically significant.

**Chemicals**

Lipopolysaccharide, 1,5-isoquinolinediol (dissolved in DMSO; final DMSO concentration in cell culture never exceeded 0.03%), 3-aminobenzamide (dissolved in ddH$_2$O), MTT- [3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide] BCIP [5-bromo-4-chloro-3-indolyl phosphate] and NBT [nitro blue tetrazolium] were from Sigma, USA. Mn(III)TBAP [Mn(III)tetrakis(4-benzoic acid) porphyrin] was from Cayman Chemicals Co., USA.

Fig. 2. (A) 1, 5-isoquinolinediol, administered 15 minutes prior to LPS (1 µg/ml) causes dose-dependent inhibition of nitrite accumulation in culture medium of J774.2 macrophages (n = 6, *p<0.05 vs. LPS group); (B) 1, 5-isoquinolinediol, administered 10 hrs after LPS (1 µg/ml) at any concentration did not influence 24-hr nitrite accumulation (n=6); (C) representative immunoblotting of NOS-2 and β-actin in LPS-treated (1 µg/ml) J774.2 cells; (D) densitometric analysis of NOS-2 protein expression. Data are normalised to β-actin levels (n = 3, *p<0.05 vs. LPS group).
RESULTS

Stimulation of J774.2 cells with LPS (1 µg/ml) caused a significant increase of 24-hr nitrite accumulation in culture medium (from 4.2 ± 0.7 to 34 ± 4 µM) (control cells were treated with vehicle, but not with LPS).

1, 5-isoquinolinediol (1-300 µM), administered 15 minutes prior to LPS dose-dependently inhibited nitrite accumulation in culture medium of J774.2 macrophages (Fig. 2A). Importantly, 1, 5-isoquinolinediol (1-300 µM), administered 10 hrs after LPS (1 µg/ml) did not influence nitrite production by LPS-treated J774.2 cells (Fig. 2B).

1, 5-isoquinolinediol, added 15 minutes prior to LPS (1 µg/ml) dose dependently inhibited NOS-2 protein induction in J774.2 cells (Fig. 2C and 2D).

Fig. 3. 3-aminobenzamide, at any concentration did not influence 24-hr nitrite accumulation in J774.2 cell culture, either administered 15 minutes prior to LPS (A) or 10 hrs after LPS (B); Neither mixture of SOD and catalase (C) nor cell permeable SOD-mimetic [Mn(III)TBAP] (D), both given 15 minutes prior to LPS (1 µg/ml) influenced 24-hr nitrite accumulation in culture medium. Data are expressed as mean ± s.e.m of n = 6 independent experiments. * p<0.05 represents significant difference as compared to LPS group.
Another PARP inhibitor, 3-aminobenzamide, at concentration up to 3 mmol/L, did not influence 24-hr nitrite accumulation in J774.2 cell culture, either administered 15 minutes prior to LPS or 10 hrs after LPS.

Interestingly, scavenging of reactive oxygen species by use of mixture of SOD and catalase (SOD/Cat, 100/300 - 1000/3000 U/ml) as well as cell permeable SOD-mimetic [Mn(III)TBAP, 1- 100 µM], both given 15 minutes prior to LPS, did not influence 24-hr nitrite accumulation in culture medium of J774.2 cells.

Neither ISO nor 3-AB affected the viability of J774.2 cells (as determined by the MTT assay) either when given alone or in combination with LPS (data not shown).

**DISCUSSION**

In this work we identified 1, 5-isoquionolinediol (ISO) as an inhibitor of induction of NOS-2 in vitro, in model of mouse macrophages stimulated with LPS.

The model of LPS-treated J774.2 cells has been widely used in studies of mechanisms of NOS-2 induction which could be easily tracked down by measurement of accumulation of nitrite in culture medium (31-33). In our hands, activation of J774.2 macrophages with LPS resulted in massive accumulation of nitrite in culture supernatant. This accumulation of nitrite clearly reflected NO production due to induction of NOS-2 in LPS-treated cells (33).

ISO dose-dependently inhibited accumulation of nitrite in supernatant of LPS-stimulated J774.2 cells. This effect was not related to its cytotoxic action (as determined by the MTT assay), but might be due to the inhibition of NOS-2 activity and/or NOS-2 induction in cells. Yet should be noted that the action of ISO was lost when the compound was given 10 hours after LPS. As in our model the stimulation of J774.2 cells for 10 hours results in nearly maximum expression of NOS-2 (data not shown), this finding shows that ISO inhibit the induction, but not the activity of NOS-2. Indeed, immunoblotting experiments have shown that ISO dose dependently inhibited NOS-2 protein expression in LPS-stimulated J774.2 cells.

The question arises about the possible mechanisms by which ISO inhibit the induction of NOS-2 in our model. In theory, there are several critical steps at which this compound may modulate cascade of molecular events leading to the expression of NOS-2 in LPS-treated macrophages. The response of the cells to LPS involves stimulation of membrane protein CD14 and toll like receptors (TLRs) with subsequent activation of downstream intracellular kinases and nuclear factors, such as nuclear factor κB (NF κB), which trigger transcription of NOS-2 gene (34). In fact, multiple pathways of NOS-2 induction seem to converge in the activation of the essential transcription factor NF κB (34,35). Interestingly, poly(ADP-ribosyl)ation may play a role in activation of NF κB (6,26,36). Thus, the inhibition of NOS-2 induction could be due to inherent property of ISO, namely inhibition of PARP. However, in our setting 3-AB,
widely used, "benchmark" inhibitor of PARP did not influence induction of NOS-2 up to 3 milimolar concentration, which was reported to inhibit PARP activity of more than 90% (27). This is why, it is tempting to speculate that inhibitory action of ISO in our model could depend on other mechanisms.

As reactive oxygen species (ROS) were shown to contribute to NOS-2 induction in LPS-activated macrophages (37), one could argue that ISO, as a compound with aromatic rings could directly counteract oxidative stress. However, this seems not to be a case in our model, as either extracellular antioxidants (SOD, Catalase) or membrane-permeable ROS scavenger [Mn(III)TBAP] did not affect NOS-2 induction. Noteworthy, this is in keeping with previous report about lack of any direct antioxidant action of ISO in vivo (38). Clearly, the exact mechanism of inhibition of NOS-2 induction by ISO requires further investigation.

There is increasing evidence of the role for poly(ADP-ribose) polymerase activation in diabetic nephropathy, neuropathy and retinopathy (39). Moreover, diabetes seems to regulate NOS-isoforms differentially by down-regulating eNOS and up-regulating iNOS (40). Recently, it was demonstrated that therapy with low-dose of ISO may efficiently reverse early peripheral diabetic neuropathy (29). Intriguingly, the concentrations of various isoquinoline derivatives in which they used to effectively inhibit PARP in cultured cells do not appear to be consistent with the unusually high efficacy of these compounds in vivo (28). Thus, the question arises, whether the beneficial action of ISO in vivo may depend, at least in part, on the inhibition of NOS-2 upregulation in diabetes? This remains attractive hypothesis to be tested.

In summary, in this study we identified 1, 5-isoquinoline as an inhibitor of induction of NOS-2 in LPS-treated mouse macrophages. However, the exact mechanism of inhibitory action of this compound requires further investigation.

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Author's address: Rafał Olszanecki, M.D., PhD, Chair of Pharmacology, 16 Grzegorzecka str, 31 531, Krakow, POLAND. Tel:+48 12 421 11 68, Fax: +48 12 4217 217.
E-mail: mfolszan@cyf-kr.edu.pl