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# PROTECTION BY PHOSPHODIESTERASE INHIBITORS AGAINST ENDOTOXIN-INDUCED LIVER INJURY IN GALACTOSAMINE-SENSITIZED MICE

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**Abstract**—Phosphodiesterase inhibitors were used as a tool to manipulate cellular nucleotide levels *in vitro* and *in vivo*. The lipopolysaccharide (LPS)-induced release of tumor necrosis factor $\alpha$ (TNF-$\alpha$) from mouse peritoneal macrophages was inhibited by prostaglandin $E_2$ with an $IC_{50}$ of 0.05 $\mu$M and by dibutyryl-cAMP with an $IC_{50}$ of 180 $\mu$M. In the presence of the phosphodiesterase inhibitors zardaverine or rolipram the intracellular cAMP concentration of LPS-stimulated macrophages was significantly increased. In these cells, LPS-inducible TNF release was inhibited by zardaverine ($IC_{50}$ = 1.5 $\mu$M) or by rolipram ($IC_{50}$ = 0.35 $\mu$M). In a model of septic shock, i.e. LPS challenge of galactosamine-sensitized mice, a dose-dependent protection against liver injury was observed following oral application of rolipram ($ED_{50}$ = 0.55 mg/kg) or of zardaverine ($ED_{50}$ ≈ 30 mg/kg). The adenylate cyclase activator forskolin was also protective. Rolipram also protected against TNF-induced liver injury in mice while zardaverine failed to do so. It is concluded that the intracellular cAMP level of macrophages is a critical determinant of LPS-inducible TNF release and therefore modulates the susceptibility to septic shock.

A rise in intracellular cAMP is associated with an attenuation of cellular activation of platelets, white blood cells, smooth muscle or lymphocytes. This was demonstrated for cell functions such as platelet aggregation [1], superoxide release [2–4], smooth muscle contraction [5–7], histamine release [8, 9], leukotriene generation [10, 11] or lymphocyte proliferation [12]. The increase of cellular cAMP levels was induced by either accelerating adenylate cyclase activity with prostaglandin (PGE‡), $\beta$2-adrenoceptor agonists, A2-adenosine receptor agonists and forskolin or by decreasing the rate of cAMP breakdown by inhibition of cyclic nucleotide phosphodiesterase (PDE).

Multiple molecular forms of PDE have been identified [13] and five families (I–V) of isoenzymes have been defined with regard to substrate specificity and substrate concentration dependence. Isoenzymes are differently distributed over various cells and tissues. It was shown that further types exist within each family [14].

A cell-specific cAMP increase by isoenzyme-selective PDE inhibitors may offer a considerable therapeutical potential. Selective PDE III inhibitors such as imazodan [15] have been established as enhancers of cardiac output with positive inotropic and chronotropic effects. PDE IV inhibitors such as rolipram or denbufylline have been demonstrated to inhibit preferentially activation of cells of nervous origin [16, 17] or inflammatory cells [18]. Activation of monocytes or macrophages of various origin was demonstrated to be suppressed in the presence of adenylate cyclase activators [19] or by PDE inhibitors [20].

In the present investigation we studied the influences of the cAMP increasing agents $PGE_2$ and forskolin, the monoselective PDE inhibitor rolipram (PDE IV) and the dual-selective zardaverine (PDE III/IV) on tumor necrosis factor (TNF) release of isolated murine macrophages. The final aim was to check the *in vivo* relevance of these *in vitro* data in a model of inflammatory liver disease which is closely related to shock.

## MATERIALS AND METHODS

*Substances. Salmonella abortus equi* lipopolysaccharide (LPS) was purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). Galactosamine was from Roth GmbH (Karslruhe, Germany). Recombinant murine TNF-$\alpha$ was a generous gift of Dr Adolf (Boehringer Institut, Vienna, Austria) or Dr Müller-Neumann (Knoll AG, Ludwigshafen, Germany). Rolipram 4-(3′-cytopentyloxy-4′-methoxypenyl)-2-pyrrolidone was from Schering AG, (Berlin, Germany) and zardaverine [6-(difluoromethoxy-3-methoxyphenyl-3-(H)] pyridazinone from Byk Gulden Lomberg Chemische Fabrik GmbH (Konstanze, Germany). Dibutyryl-cAMP (db-cAMP), forskolin, streptomycin, penicillin, and heparin were purchased from Sigma. $PGE_2$ was a gift by Dr Entries (Upjohn, Heppenheim, Germany). The radioimmunoassay for determination of cAMP was from Amersham (Braunschweig Germany).

*Isolation of mouse peritoneal macrophages.* Cells were isolated essentially as described previously [21] after lavage of the peritoneal cavity with 5 mL of

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‡ Abbreviations: LPS, lipopolysaccharide, i.e. endotoxin; db-cAMP, (dibutyryl) cyclic adenosine monophosphate; (rmu)TNF, (recombinant murine) tumor necrosis factor-$\alpha$; GalN, D-galactosamine; ALT, alanine aminotransferase; PDE, phosphodiesterase; $PGE_2$, prostaglandin $E_2$; DMSO, dimethyl sulfoxide.

RPMI 1640 medium (Biochrom, Berlin, Germany) containing 1 μg/mL penicillin, 100 U/mL streptomycin and 2.5 U/mL heparin. Cells were centrifuged at 500 *g*, 4°, 10 min, then resuspended in RPMI lacking heparin and containing additionally 10% fetal calf serum. Cells were seeded in 96 well plates in a density of $2 \times 10^5$ per well in 200 μL. They were allowed to adhere during 2 hr. Non-adhesive cells were removed by washing twice with RPMI medium. Staining (according to Ref. 22) identified 84 ± 3% of the cells as macrophages. Vitality at the beginning of the incubation were estimated to be 95 ± 2% and 90 ± 4% at the end using Trypan blue exclusion.

Cells were incubated in 250 μL of RPMI 1640 medium and the test compounds were added as a solution in dimethyl sulfoxide (DMSO) yielding a final DMSO concentration of 0.7%. The solvent controls contained 0.7% DMSO only.

*Assessment of* D-*galactosamine (GalN)/LPS-induced liver injury* in vivo. Male NMRI-mice (25–35 g body weight) were kept at least 1 week on a standard diet (Altromin 1310) *ad lib*. Hepatitis was induced by GalN/LPS or GalN/TNF-α as described previously [23]. Briefly, the animals were injected i.p. with galactosamine (700 mg/kg) plus LPS (30 μg/kg). Alternatively, the GalN-sensitized animals were challenged by i.v. injection of 15 μg/kg recombinant murine TNF-α[(rmu)TNF] 60 min after GalN. Test compounds were given by oral gavage in a volume of 10 μL/g body weight. Rolipram was administered as a suspension in cremophor EL (Sandoz AG, Basel, Switzerland). Zardaverine was dissolved in 1,2 propylenglycol, 1 N NaOH, water in a ratio of 1:1.4:67.3 and ultrasonicated for 5 min at 65°. Forskolin was dissolved in cremophor EL (7% of the final volume) and then diluted with pyrogen-free phosphate-buffered saline. No signs of acute toxicity were observed in mice with up to 50 mg/kg of rolipram or zardaverine. Blood samples were taken 8 hr after intoxication (LPS, i.p.) by cardiac puncture after cervical dislocation. Liver injury was assessed using serum alanine aminotransferase (ALT) [24].

*TNF assay*. TNF was measured in serum of the animals taken by retro-orbital puncture 90 min after challenge or cell supernatants 3 hr after incubation. TNF was determined using a bioassay performed with the fibrosarcoma cell line WEHI 164 clone 13 according to Espevik and Nissen-Meyer [25].

*Measurement of cellular cAMP*. The effects of rolipram and zardaverine upon cellular levels of cAMP were determined in LPS-stimulated murine peritoneal macrophages. Macrophages ($2 \times 10^5$) were preincubated in 96-well microtiter plates with rolipram (100 μM) or zardaverine (100 μM) 1 hr prior to LPS addition (1 μg/mL). Thirty minutes after addition of LPS the reaction was terminated with trichloroacetic acid (TCA) (5% end concentration). The supernatants were centrifuged (10 min, 10,000 *g*), and the samples were neutralized with freon and trioctylamin (1:1, 200 μL). cAMP was measured by radioimmunoassay kits ([$^3$H]cAMP) purchased from Amersham.

*Statistics*. All *in vitro* experiments were performed in triplicate. *In vivo*, groups of six to eight were used. Statistical analysis was performed using the Student's *t*-test, where $P < 0.05$ was considered to be significant.

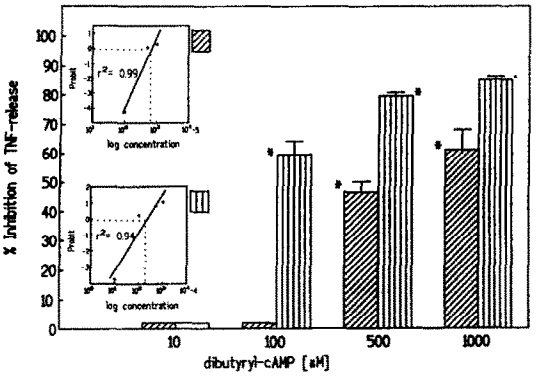


Fig. 1. Concentration dependence of the inhibition of TNF-release from mouse peritoneal macrophages 4 hr after stimulation by 1 μg/mL LPS. Diagonal bars represent experiments where db-cAMP was added simultaneously with LPS, vertical bars where db-cAMP was added 30 min prior to LPS. Insets show the probit analysis of the experiments. Data ± SEM, N = 3, *P ≤ 0.05 vs LPS control.

## RESULTS

Previous studies had shown that addition of exogenous $PGE_2$ led to a dose-dependent reduction of the release of TNF following a LPS stimulus from murine macrophages [19]. On the other hand, since it was demonstrated that $PGE_2$ increases intracellular cAMP [20], we first of all carried out experiments in our cells suitable to relate these events.

When mouse peritoneal macrophages were incubated in the presence of varying amounts of $PGE_2$, a dose-dependent attenuation of TNF release as determined after 4 hr was observed with a 50% inhibition at a concentration of 0.05 μM (data not shown). This inhibition was only seen when $PGE_2$ was added simultaneously with LPS while no inhibition was seen when prostaglandin was added 30 min prior to LPS. This finding indicates that a fast rise of cAMP following LPS may be essential for the inhibition of TNF release. In the following experiments, the permeable analog dibutyryl-cAMP was directly added to mouse peritoneal macrophages stimulated with LPS either 30 min prior to or simultaneously with stimulant. Also under these conditions, a dose-dependent attenuation of LPS-induced TNF release was observed with an $IC_{50}$ of 180 μM (Fig. 1). These experiments confirm previous findings that exogenously added cAMP is able to block TNF release [21]. It is further demonstrated that the rise in cAMP occurs within a narrow time window after addition of LPS.

In the following experiment the intracellular cAMP concentrations were measured in LPS-stimulated cells under various conditions. The data

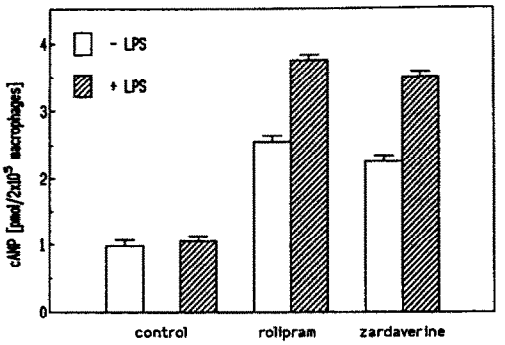


Fig. 2. Elevation of the intracellular cAMP concentration in murine peritoneal macrophages by rolipram or zardaverine in the presence (hatched bars) or absence (open bars) of LPS. Macrophages ($2 \times 10^5/200\ \mu L$) were preincubated with rolipram (100 $\mu$M) or zardaverine (100 $\mu$M) 1 hr prior to LPS addition (1 $\mu$g/mL). Intracellular cAMP concentrations were determined 30 min after LPS addition. Data represent mean values ± SD, experiments were carried out in triplicate.

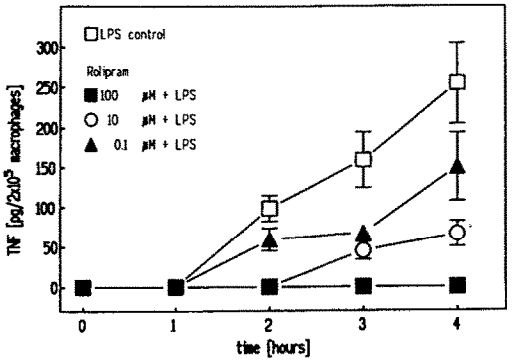


Fig. 3. Inhibition of the LPS-induced TNF release from murine peritoneal macrophages by rolipram. Macrophages ($2 \times 10^5/200\ \mu L$) were preincubated with rolipram for 1 hr at 37° prior to LPS addition (1 $\mu$g/mL). Controls were treated with LPS only (1 $\mu$g/mL). After various times the TNF concentrations were measured in the supernatants using the bioassay described in Materials and Methods. Data represent mean values ± SEM, experiments were carried out in triplicate.

in Fig. 2 show that within 30 min, the addition of LPS to mouse peritoneal macrophages had no significant influence on the intracellular cAMP level. However, at an arbitrarily chosen test concentration of 100 $\mu$mol/L the PDE inhibitors zardaverine and rolipram either alone or in the presence of LPS led to an approx. 3-fold increase of intracellular cAMP within this time. This finding demonstrates *in vitro* that these two PDE inhibitors increase the steady-state concentration of cAMP. The next experiment addressed the functional consequences of this pharmacological cAMP enhancement: since macrophages when stimulated with LPS start to release TNF into the medium after 1 hr, we investigated the TNF release of these cells in the presence and absence of varying concentrations of PDE inhibitors. The time course displayed in Fig. 3 demonstrates that rolipram dose-dependently attenuated the release of TNF from LPS-stimulated cells. At a fixed time, i.e. 4 hr after LPS, a half-maximal inhibition by rolipram was found at 0.35 $\mu$M, while a value of 1.5 $\mu$M was obtained for zardaverine (Fig. 4). These experiments indicate that enhanced intracellular cAMP levels are likely to be the cause for the inability of these cells to produce or release TNF-$\alpha$.

The second part of this investigation addressed the question of the *in vivo* relevance of the described *in vitro* data. In order to answer this question, *in vivo* experiments were performed in a model where the systemic presence of LPS-inducible TNF is known to be a central pathogenic process [26–28]. The data in Table 1 show that rolipram, as well as zardaverine, pretreatment of mice led to a dose-dependent protection against LPS-induced hepatitis (left column). The data in the right-hand column demonstrate that the amount of TNF found in sera of these animals 1 hr after LPS, i.e. in the peak of the TNF release, was dose-dependently suppressed by either drug. These findings provide *in vivo* evidence that PDE inhibitors are able to block the

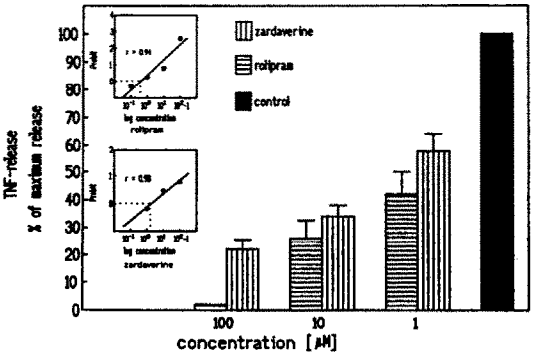


Fig. 4. Concentration dependence of the inhibition of TNF-release from mouse peritoneal macrophages by phosphodiesterase inhibitors 4 hr after stimulation with 1 $\mu$g/mL LPS. Horizontally hatched bars represent experiments where dibutyryl-cAMP was added simultaneously with LPS, vertically hatched bars are experiments where dibutyryl-cAMP was added 30 min prior to LPS. Insets show the probit analysis of the experiments. Data are means ± SEM, N = 3, *P < 0.05 vs LPS control.

LPS-inducible TNF release into the circulation and thus prevent TNF-mediated liver injury.

A final experimental approach was made by comparing the *in vivo* efficacy of PDE inhibitors against hepatitis induced either by administration of LPS, or alternatively by direct i.v. administration of TNF-$\alpha$. The contents of Table 2 show that zardaverine fully protected against LPS-induced hepatitis in galactosamine-sensitized mice and failed to do so when hepatitis was induced by TNF-$\alpha$. In contrast, administration of rolipram protected in either model. Since this finding was surprising, we

Table 1. Dose-dependent protection by oral rolipram or zardaverine pretreatment against GalN/LPS-induced hepatitis in mice

| Pretreatment | ALT [U/L]† | TNF [ng/mL]‡ |
|---|---|---|
| Hepatic injury control | 3660 ± 1580 | 41 ± 21 |
| None | 80 ± 20* | N.D. |
| Rolipram 5.50 mg/kg | 110 ± 20* | 0.7 ± 0.2* |
| Rolipram 0.55 mg/kg | 940 ± 350 | 13 ± 5 |
| Rolipram 0.15 mg/kg | 2490 ± 940 | 17 ± 8 |
| Solvent§ | 3840 ± 1640 | 33 ± 17 |
| Zardaverine 68 mg/kg | 110 ± 30* | 0.2 ± 0.1* |
| Zardaverine 27 mg/kg | 1050 ± 350 | 6 ± 3 |
| Zardaverine 14 mg/kg | 2200 ± 1270 | 18 ± 13 |
| Solvent§ | 3090 ± 1580 | 20 ± 12 |

* $P \leq 0.05$ vs GalN/LPS control.

† Plasma ALT was determined 8 hr after administration of GalN/LPS, aspartate aminotransferase and sorbitol dehydrogenase data were analogous to ALT values.

‡ Serum TNF was determined 1 hr after administration of GalN/LPS.

§ Solvent control group animals were treated with rolipram-solvent (cemophor and 0.9% NaCl, 1:4) or zardaverine/solvent (1.2 propylenglycol, 1 N NaOH and $H_2O$, 1:1.5:67) 1 hr prior to GalN/LPS. Doses and application routes: GalN (700 mg/kg) was administered simultaneously with 5 μg/kg LPS i.p., rolipram or zardaverine were administered p.o. 1 hr prior to GalN/LPS.

Number of animals per group = 5–12, data ± SEM, N.D. = not detectable.

Table 2. Differential effect of rolipram, zardaverine or forskolin pretreatment in male GalN-sensitized mice against hepatitis induced by either LPS or by TNF

| Pretreatment | GalN/LPS ALT [U±L]† | GalN/TNF-α ALT [Y±L]† |
|---|---|---|
| Hepatic injury control | 4750 ± 1575 | 2900 ± 1185 |
| Zardaverine 68 mg/kg | 110 ± 30* | 5180 ± 1100 |
| Rolipram 5.5 mg/kg | 110 ± 20* | 80 ± 20* |
| Forskolin 10 mg/kg | 140 ± 20* | 20 ± 10* |

* $P \leq 0.05$ vs hepatic injury control.

† Plasma ALT was determined 8 hr after application of GalN/LPS or TNF-α, aspartate aminotransferase and sorbitol dehydrogenase data were analogous to ALT values. The solvent control groups and untreated animals had concentrations of transaminases below 150 U/L.

Dose and application routes: GalN (700 mg/kg) was administered simultaneously with 5 μg/kg LPS i.p., 15 μg/kg rmu-TNF-α was i.v. injected 1 hr after 700 ng/kg GalN (i.p.). Rolipram or zardaverine were administered p.o. 1 hr before GalN/LPS or TNF-α. Forskolin was i.p. injected 15 min prior to GalN/LPS or 30 min to TNF-α.

Number of animals per group = 5–12, data ± SEM.

extended this experiment by checking the *in vivo* effect of forskolin, a tool known to increase cAMP levels in organs and cells. Like rolipram, administration of 10 mg/kg of forskolin fully protected the animals against either challenge (Table 2, last line). When the time-dependence of this protection was studied, it was found that forskolin was able to protect significantly against TNF-induced hepatitis in galactosomine-sensitized mice only when given 30 min prior to TNF challenge. Administration of forskolin 15 min or 45 min prior to TNF administration failed to protect (data not shown). These findings indicate that only within the narrow time-frame, the enhancement of intracellular cAMP by various means results in an impairment of TNF production and/or release accompanied by protection against TNF toxicity.

## DISCUSSION

Septic shock induced by translocation of gram-negative bacteria or their components into the circulation is still a clinical problem which is hardly managed. It can be experimentally studied by infusion of LPS into animals such as sheep or rats and leads to the typical multiorgan failure under these pathological conditions [26]. Alternatively, the organ-specific sensitization of the liver by co-administration of galactosamine can be used to study the septic shock syndrome in mice under conditions where a single organ failure, i.e. a fulminant hepatitis, is the pathological end-point [23, 27]. In either model, the release of pathogenic mediators by mononuclear cells is triggered by LPS. The propagation of these mediators includes leukotrienes, particularly the extremely potent vasoconstrictor $LTD_4$, the release of superoxide and its potency to inactivate systematically present antiproteases such as $\alpha_1$-antitrypsin and finally the proteolytic release of a TNF-α precursor expressed on the surface of the monocytic cell following LPS exposure [28]. Thus, TNF in the circulation represents the pivotal and terminal mediator of septic shock.

Infusion of LPS into the blood of sheep was shown to induce changes in the release of cyclic nucleotides into the circulation without an identification of cellular origin of these products [29]. On the other hand, various studies have been reported where changes in cellular cyclic nucleotide levels modulated the release of inflammatory mediators including oxygen radicals, platelet activation factor and cytokines [4, 11, 19]. Since the cause and effect of relationships between cyclic nucleotide changes in effector cells and possible consequences to target cells were unclear, we decided to use PDE inhibitors as pharmacological tools to study this issue *in vivo*.

The first part of our study confirms *in vitro* that cAMP levels can be efficiently modulated in the presence of various PDE inhibitors. The functional consequence of elevated cAMP levels in mouse peritoneal macrophages was a dose-dependent attenuation of TNF release. If this *in vitro* finding translates into the *in vivo* situation, then a protection against a pathological condition induced by TNF release has to be expected. This was actually the case. As a negative control experiment, it was shown that a similar treatment at similar doses failed to protect against i.v. administered TNF-α in the case of zardaverine. This control experiment contains further information. It is known that exposure of animals to low, subtoxic doses of LPS leads to a

state of tolerance against a second toxic treatment with endotoxin [30]. Therefore, care has to be taken in the interpretation of experiments such as ours in order to exclude a possible protection which might have been due to induction of tolerance. The known cross-tolerance between LPS and TNF and the finding that no protection was found against TNF with zardaverine makes this possibility unlikely.

In the case of a rolipram treatment, different results were obtained compared to those with zardaverine. Clearly, rolipram was able to protect also against TNF-induced hepatotoxicity in galactosamine-sensitized mice. Since this drug was given orally, the tolerance induction as a possible misinterpretation does not apply. Our results do not allow one to decide whether these different pharmacological qualities of the two drugs are due to their different capabilities of inhibiting various phosphodiesterases or whether pharmacokinetic and distribution phenomena are responsible for this effect. Either PDE inhibitor bears the potential of being used as a preventive means of inhibiting the LPS-induced TNF release and consequently of preventing septic shock.

Our interpretation that the intracellular cAMP level is the critical determinant for regulating the LPS-induced TNF release, is further supported by a different pharmacological approach used in this study, i.e. by activating adenylate cyclase with forskolin. Treatment of mice with forskolin protected mice not only against LPS-induced hepatotoxicity via suppression of TNF reduction but also within a short time-frame against a TNF challenge as such. These findings suggest that not only the effector cells but also the target cells might be prone to a regulation of their susceptibility against inflammatory mediators via cyclic nucleotide increase. This aspect which appears clinically relevant as a preventive protection, might allow development of curative drugs against septic shock but requires further investigation.

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