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PENTOXIFYLLINE DOES NOT ACT VIA ADENOSINE RECEPTORS IN THE INHIBITION OF THE SUPEROXIDE ANION PRODUCTION OF HUMAN POLYMORPHONUCLEAR LEUKOCYTES

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Summary: The inhibitory effect of adenosine (ADO) and pentoxifylline (POF) was studied alone and in combination on the N-formyl-methionyl-leucyl-phenylalanine (FMLP) stimulated superoxide anion production of human polymorphonuclear leukocytes (PMNL). The pharmacological analysis of the results of these experiments demonstrated greater than additive and independent interaction of the drugs, representing potentiation. These results reflect differences between the sites of action of ADO and POF. Accordingly, the ADO receptor antagonist 8-phenyltheophylline only diminished the inhibition mediated by ADO, but totally failed to affect POF. Therefore, we hypothesize that POF acts as a phosphodiesterase inhibitor, potentiating the increase in cyclic AMP induced by ADO due to the stimulation of the adenylate-cyclase of human PMNL.

The nucleoside ADO has been shown to inhibit several leukocyte functions, such as the production of superoxide anions, degranulation, adherence, cell-mediated cytotoxicity and can enhance the chemotactic response of PMNL (12). These pharmacodynamic effects are also shared by POF. ADO acts via specific receptors on the outer surface of the PMNL leading to the activation of the adenylate-cyclase-system (6). In contrast, the action of POF has not yet been fully understood. In general, two modes of action might be considered in the inhibition of leukocyte function. While the methylxanthine POF could act as an inhibitor of phosphodiesterases, it is also possible that POF interferes with the specific ADO receptors. This latter possibility was supported by the findings that the specific ADO receptor antagonist BW A1433U was able to block POF in restoring the chemotactic response of human PMNL after inhibition by TNF-α (16). In order to elucidate these mechanisms, the effects of POF and ADO were investigated for each drug alone and in combinataion on the superoxide anion production in FMLP - stimulated PMNL. The dose-response curves obtained were

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analyzed by the pharmacological data evaluation method described by PÖCH et al. (9) that allows to differentiate the pharmacologic sites of action of drugs with similiar effects. In addition, the effect of the ADO receptor antagonist 8-phenyltheophylline was tested for the ADO - and POF - mediated inhibition, in comparison.

Material and Methods

Reagents. Adenosine (ADO), pentoxifylline (POF), 8-phenyltheophylline (8-PT), N-formyl-methionyl-leucyl-phenylalanine (FMLP), lucigenin, superoxide dismutase (SOD, EC 1.15.1.1) and cytochalasin B were obtained from Sigma Biochemical Co. (St.Louis). Ficoll-Hypaque 400 (density 1.077) was from Biochrom (Berlin, F.R.G.). All other chemicals were of reagent grade.

Isolation of human polymorphonuclear leukocytes was performed on freshly drawn blood of healthy volunteers by Hypaque-Ficoll gradient and dextran sedimentation. Isolated cells were washed three times in Hanks-buffered-salt-solution and kept in glass tubes at $4^{o}C$ after resuspension until use. Purity and viability of PMNL were more than 95%.

Superoxide anion production of PMNL was measured by lucigenin-enhanced chemiluminescence (14). The reaction mixture contained 10^5 PMNL and different agonists and was incubated in polystyrene cuvettes (10 min, $37^{o}C$, final volume 500 µl) in the Biolumat counting chamber (Biolumat model 9505, Berthold, Wildbad, FRG). Thereafter, FMLP was injected and the final concentrations of the components were as followed: FMLP 1×10^{-7} M, lucigenin 1×10^{-4} M, cytochalasin B 5×10^{-4} g/l, ADO 5×10^{-8} - 1×10^{-3} M, POF 10^{-6} - 10^{-2} M, 8-PT 5×10^{-6} M. The specifity of lucigenin to detect superoxide anions was controlled in the presence of SOD (5µg/ml) that completely abolished the chemiluminescence activity. Heat inactivated SOD was without any effect on the chemiluminescence.

Data evaluation and statistics. The combined drug effects were evaluated by the method of PÖCH et al. (9). In brief, all data were expressed in terms of percent inhibition of control activation and the median values were calculated. Advantage was taken from the curve-fitting programm ALLFIT to construct the dose response-curves for median values. From these observed data, theoretical curves were calculated, assuming either effects for additive or independent modes of drug interaction. The chi-square of goodness-of-fit test was performed by the computer program STATGRAPHICS to test for significant differences between the observed values and the calculated curves.

Results

Figure 1 shows the inhibition of the chemiluminescence activity of PMNL by increasing concentrations of ADO or POF. As can be seen, the dose-response curves for ADO and POF were both sigmoidal-shaped reaching maximal values at 10^{-6} M for ADO and 10^{-2}M for POF. The half-maximal inhibitory concentration (IC_{50}) was about 10^4 times lower for ADO than for POF ($IC_{50} \approx 1 \times 10^{-7}$ M for ADO vs. $IC_{50} \approx 1 \times 10^{-3}$M for POF). When single concentrations of POF were combined with ADO, POF dose-dependently increased the inhibition mediated by ADO (Fig. 2A). In order to characterize the mechanism of the drug interaction, the theoretical dose-response curves were calculated for the respective drug combinations assuming either an additive (Fig. 2B) or an independent drug interaction (Fig.

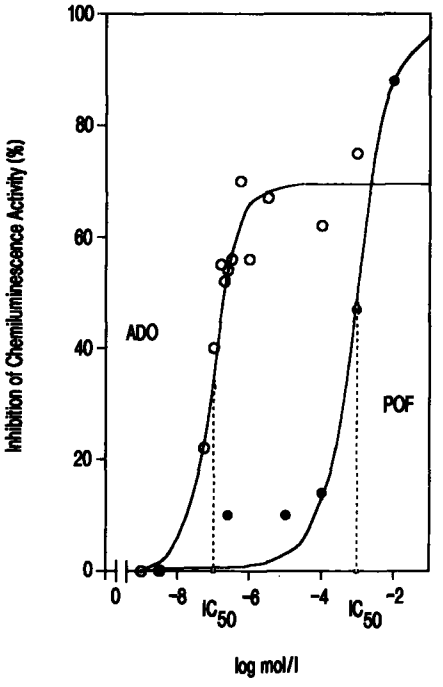

Figure 1. Fitted dose-response-curves of the observed effects of ADO or POF on the FMLP-induced O_2^- production of human PMNL. PMNL (1×10^5) were stimulated with FMLP (1×10^{-7} M) in the presence of increasing concentrations of ADO or POF. Control chemiluminescence activity was 19×10^3 cpm / 10^5 PMNL. (Median, n = 6 - 8).

2C). As can be seen, the inhibition experimentally observed for the combination of ADO and POF at 10^{-4} M or 10^{-3} M was significantly above that expected in the case of an additive as well as an independent drug interaction. When the ADO receptor antagonist 8-PT was tested, only the inhibitory effect of ADO was antagonized, while POF was not affected (Fig.3).

Discussion

POF has been proven to be an effective therapeutic tool in the prevention and therapy of experimentally-induced multiple organ failure. In vitro studies demonstrated an inhibition of the release of cytotoxic agents from stimulated leukocytes and an increase in the directed migration. POF was effective even on those PMNL that had been primed before with cytokines (16). Although a lot has been learned about the effects of POF on particular cell functions, up to now only little is known about the biochemical pathways mediating these effects. Several studies demonstrated an increase of cyclic AMP associated with a decrease of intracellular calcium suggesting an interference of POF with the formation of second messengers in PMNL (15,16). Interestingly, these pharmacodynamic effects are also shared by ADO, because this nucleoside can

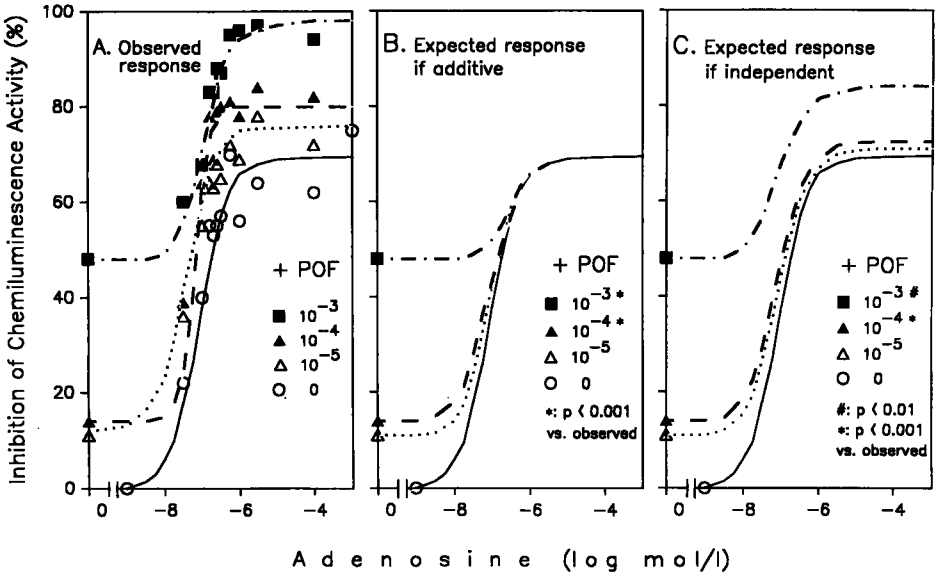

Figure 2.

A. Fitted dose-response-curves of the observed effects of ADO in the abscence and presence of increasing concentrations of POF. The observed effects exerted by the different concentrations of POF alone are indicated by the respective symbols on the ordinate. PMNL (1×10^5) were stimulated with FMLP (1×10^{-7} M). (Median, n = 6 - 8.)

B. Dose-response-curves calculated for an additive drug interaction of ADO and POF. The expected additive dose-response-curves describe the effect of POF and ADO acting at the same molecular site.

C. Dose-response-curves calculated for the independent drug interaction of ADO and POF. The expected independent dose-response-curves describe the combined effects of drugs acting at different sites and independently of each other.

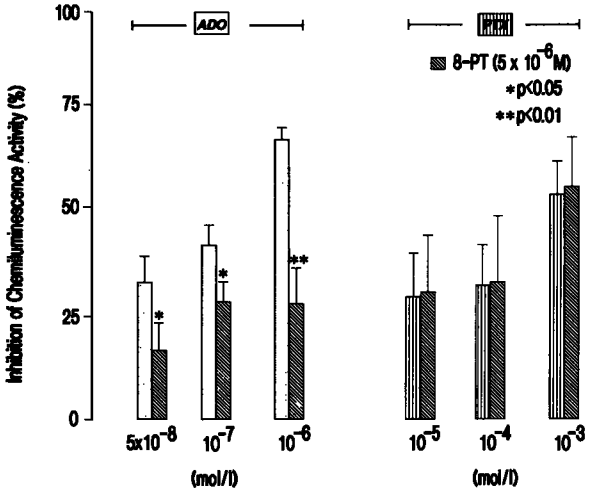

Figure 3. Effect of the A1/A2-receptor antagonist 8-phenyltheophylline (8-PT) on the ADO - and POF - mediated inhibition of the chemiluminescence activity. PMNL (1×10^5) were stimulated with FMLP (1×10^{-7} M) in the presence of ADO or POF and 8-PT. (Mean ± S.E., n = 6-8, paired t-test.)

inhibit the production of reactive oxygen metabolites and can enhance chemotaxis. In addition, the close relationship between the increase in cyclic AMP and the decrease in intracellular calcium was also demonstrated for ADO (7,17). Therefore it is conceivable that both substances can effectively inhibit the leukocyte functions by the same messengers, cyclic AMP, but by different molecular sites of action. There is clear evidence from our experimental studies that ADO and POF act at different sites in the inhibition of the superoxide anion formation. Wherease the inhibitory action of ADO could be diminished by the ADO-receptor antagonist 8-phenyltheophylline (13), the phenomenologically similar action of POF was not affected by this antagonist (Fig.3). When ADO and POF were combined, the drugs acted in an overadditive, synergistic way. This result strongly supports different sites of pharmacologic action, because drugs that act at the same pharmacologic site are characterized by an additive interaction (9). Moreover, the combined actions significantly exceeded the values calculated for an independent drug interaction, demonstrating the phenomenon of drug potentiation (9).

From these results, demonstrating an overadditive potentiation of ADO by POF, it can be concluded, that:

i) POF does not act as an uptake inhibitor of ADO. In this case POF would mediate its effects just by increasing the concentration of extracellular ADO. Under these conditions no increase in the maximum effect of ADO, but a left shift of the dose-response curves from the additive curves would be expected. This was not the case. In Fig.2B the calculated additive dose-response-curves are shown, when POF is assumed to act as an equi-effective dose of ADO. In agreement with our findings are the results of Hand et. al. (4), reporting no effect of POF on the uptake of ADO in unstimulated human PMNL. In addition, the enzyme adenosine deaminase failed to alter the inhibiton of superoxide generation mediated by POF.

ii) POF potentiates ADO most likely by the inhibition of the enzyme phosphodiesterase, known to degrade cyclic AMP, the formation of which is stimulated by ADO acting via A2-receptors on the adenylate-cyclase-system in human PMNL (2,6,7). This explanation fits well with the phenomenon of drug potentiation, which is characteristically obtained with drugs that are metabolically linked by one common product, the formation of which is stimulated by one drug and is enhanced by the inhibition of its degradation by the other drug (10,11). For instance, when the adenylate-cyclase-stimulator isoprenaline was combined with the phosphodiesterase-inhibitor papaverine, the drugs potentiated each other in the relaxation of isolated bovine coronary arteries (9,10). In addition, the observed dose-response curves of ADO in the presence of POF (Fig. 2) resemble those of VIP or secretin in the presence of various phosphodiesterase-inhibitors (3).

However, it can not be ruled out, that POF increases the efficacy of the ADO receptor system, since another methylxanthine - derivative (isobutylmethyl-xanthine) was shown to enhance the signal-transduction mechanisms by its action on G-proteins (8).

Noteworthy, the drug potentiation reported here may be of clinical significance, particularily in humans with septic shock. These patients are generally suffering from an imbalance in the supply-demand ratio of oxygen. The oxygen deficit, however, is the driving force for the degradation of energy-rich adenine-nucleotides, resulting in the enhanced formation of adenosine. In agreement with this, BARDENHEUER et al. (1) demonstrated five fold higher plasma levels of ADO in patients with septic shock than in healthy volunteers. Therefore, POF may exert its beneficial therapeutic effects in the septic state by the potentiation of endogenously formed ADO. This the more, because the concentrations of POF tested in our study were also achieved in man during oral application of POF at 3 x 400 mg per day, resulting in steady state plasma levels of POF and its active main metabolite M1 of about 1×10^{-5} - 1×10^{-4} M (5).

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