**Pseudomonas aeruginosa** exotoxin A induces pulmonary endothelial cytotoxicity: protection by dibutyryl-cAMP

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ABSTRACT: In pseudomonal septicaemia, serum levels of antibody to exotoxin A have been demonstrated to be an important independent predictor of survival. Previously, we have demonstrated that exotoxin A directly injures pulmonary endothelial cells, and that dibutyryl-cyclic adenosine monophosphate (Db-cAMP) can attenuate this injury. The object of this study was to examine the mechanisms of this pulmonary endothelial cell injury and the mechanism of Db-cAMP protection. The effects of differing duration of exposure to exotoxin A and a reduction in temperature on endothelial cell injury were examined. In addition, the effect of post-treatment with Db-cAMP on exotoxin A-induced endothelial cell injury was studied.

A brief, 5 min, exposure to exotoxin resulted in maximum injury comparable to that produced by 18 h exposure. This injury did not occur at low temperatures, which would inhibit receptor-mediated endocytosis. Db-cAMP protected endothelial cells, even when added up to one hour after exotoxin exposure.

These results suggest that, in this model, exotoxin A-induced injury of endothelial cells is receptor-mediated. Furthermore, this injury may be attenuated even after exotoxin A internalization has taken place.


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Cell Culture

Standard tissue techniques were used to grow BPAECs in RPMI media containing 20% heat inactivated foetal calf serum with 2 mM glutamine, penicillin 100 U·ml\(^{-1}\), and streptomycin 200 U·ml\(^{-1}\). Experiments were performed on cells between passage numbers 18–25. Trypsin (0.05%) was used to release cells from the flasks. Ethidium bromide staining confirmed that the cells were >98% viable before plating on 24 well plates at a density of 10\(^5\) cells·well\(^{-1}\).

Chromium (Cr-51) assay

When the cells were near confluence (80–90%), Cr-51 was added overnight at a concentration of 2 µCi·ml\(^{-1}\). After this labelling process, excess chromium was washed off with HBSS. Subsequent Cr-51 release to the overlying media was then measured to assess cell damage [12, 13]. The total possible Cr-51 counts (total) were determined from the addition of control well cell lysates plus the overlying media counts. The cells were lysed by adding 1 ml of NH\(_4\)OH to the well for 10 min. Injury to the cells for each experimental condition was then calculated, using the overlying media in the following manner, as described by KAMP et al. [14]: % specific Cr-51 release = (test media counts per minute (cpm) - control media cpm/total cpm) \(\times\) 100. This calculation corrects for spontaneous Cr-51 release from control wells. The spontaneous %Cr-51 release over 18 h from control wells was 31±2.6% (mean±SEM) of the total releasable Cr-51 (n=6).

Exposure of BPAECs to Exo A

Exo A (10 µg·ml\(^{-1}\)) was added to confluent monolayers of Cr-51-labelled cells in 24 well plates, for times varying from 5 min to 18 h. At the completion of exposure to Exo A, the overlying media (1 ml) was removed, the monolayer rinsed, and fresh media was added to the monolayer. At 18 h from the initial exposure to Exo A, the Cr-51 counts were determined. Each experimental condition was repeated in duplicate. Experiments were also carried out to examine the effects of low temperature (4°C) on Exo A injury over an 18 h time period.

Dosage of Db-cAMP

The optimum dose of db-cAMP was determined by varying the dosage between 0.1, 1, and 10 mM with a standard dose of Exo A (10 µg·ml\(^{-1}\)). The optimal dose of Db-cAMP was then added immediately before Exo A and subsequently at various time-points after Exo A, ranging from 5 min to 4 h, to determine how long after Exo A addition Db-cAMP might confer protection.

Statistics

The experiment results are expressed as the mean±SEM. The Student’s t-test was used to compare between two groups. Where there were multiple groups, one way analysis of variance (ANOVA) for time and treatment effects was performed, followed by the Neuman-Keuls range test. A p-value of less than 0.05 was selected as significant.

Results

Effect of Exo A exposure time to endothelial cell injury

Exposure of BPAECs to Exo A for various periods of time indicated that there was no difference in the level of injury whether the endothelial cells were exposed to Exo A for 5 min or 18 h in the media, although there was a trend for chromium release to increase slightly with time. There was 26±3% specific Cr-51 release at 5 min and 33±3% specific Cr-51 release at 18 h (n=6) (fig. 1).

Effect of temperature on Exo A injury to endothelial cells

When experiments were carried out at 4°C, the endothelial cells incubated with Exo A for 18 h demonstrated significantly less Cr-51 release than endothelial cells incubated with Exo A at 37°C (0.5±2.8 4°C vs 33.0±2.5 37°C; p<0.05)

![Fig. 1.](image-url) - The effect of exposure time to exotoxin A (Exo A) on BPAEC injury. BPAECs were exposed to Exo A (10 µg·ml\(^{-1}\)) for time periods from 5 min to 18 h. There were no differences in Cr-51 release between any of the exposure times. Results are expressed as the mean±SEM, n=6. BPAEC: bovine pulmonary artery endothelial cells.
We demonstrated that 1 mM Db-cAMP was the optimum dose conferring protection to endothelial cells against a given dose (10 µg·ml⁻¹) of Exo A (fig. 2a). A 10 mM dose of Db-cAMP did not confer any protection, and 10 mM alone actually caused significant Cr-51 release when compared to control and 1 mM Db-cAMP alone (fig. 2b). Db-cAMP was protective when co-incubated with Exo A. Specific Cr-51 release was determined to be 10±2% in this case.

This protective effect of Db-cAMP against Exo A-induced endothelial injury was also noted with post-incubation of Db-cAMP up to 1h after Exo A. If Db-cAMP was added to the wells 1 h after Exo A, the specific Cr-51 release was 13±3% (n=6). The lowest level of specific Cr-51 release was demonstrated when Db-cAMP was added 5 min post-Exo A, where specific Cr-51 release was 4±2% (fig. 3).

Our results establish that Exo A-induced pulmonary endothelial cell injury is independent of the duration of exposure to Exo A and can be prevented by temperature reduction. In this model, it appears that after one hour of exposure to exotoxin, irreversible injury to endothelial cells has occurred. These observations support the findings of FITZGERALD et al. [10], in mouse fibroblasts, that Exo A is taken up by receptor-mediated endocytosis. In our model, binding of Exo A to the cell surface occurs rapidly, as removal of the Exo A following 5 min of exposure did not prevent the occurrence of injury. Our experiments do not clarify whether endocytosis also occurs rapidly, or whether this requires more time. The former observation has important clinical implications, as early interventions, such as monoclonal antibody binding to toxins, may be unhelpful if the toxin has already been bound to the cell surface and possibly been internalized by endocytosis.

We have also demonstrated that the addition of Db-cAMP is protective if added up to one hour after Exo A. In contrast to other studies of lung injury, we were not measuring endothelial cell permeability but severe cell damage as related to Cr-51 release, which is a late event [15]. Consequently, this post-treatment cytoprotection is more significant and is consistent with in vivo work of HOFFMANN et al. [16], who demonstrated that Db-cAMP and pentoxiphylline attenuated endotoxin-induced lung injury in guinea-pigs in vivo when added 30 min post-endotoxin. Db-cAMP has been demonstrated to attenuate lung injury from a number of injurious stimuli [17–21]. However, the mechanism of protection in these instances is not known, although there is evidence that in some in vivo models it may exert a beneficial response by reducing pulmonary vascular pressures [19, 20]. However, there is also evidence that this protection may be related to decreasing endothelial permeability [20, 21]. A further hypothesis to explain the protective influence of

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**Fig. 2.** a) The optimum dose of Db-cAMP against Exo A injury (10 µg·ml⁻¹) to BPAECs. The optimum dose of Db-cAMP was determined to be 1 mM Db-cAMP. : 10.0 mM Db-cAMP + Exo A; : Exo A alone; : 0.1 mM Db-cAMP + Exo A; : 1 mM Db-cAMP + Exo A. b) 1 mM Db-cAMP did not cause significant Cr-51 release, although 10 mM caused significant Cr-51 release. : control; : control + 1 mM Db-cAMP; : control + 10 mM Db-cAMP. *: p<0.05 compared to control wells. Results are expressed as the mean±SEM, n=4. Db-cAMP: dibutyryl cyclic adenosine monophosphate. For further abbreviations see legend to figure 1.

**Fig. 3.** Post-treatment with Db-cAMP attenuates Exo A (10 µg·ml⁻¹) injury. Results are expressed as the mean±SEM, n=6. *: p<0.05 as compared to Exo A alone. For abbreviations see legend to figure 1.

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Db-cAMP on pulmonary endothelial cell injury is that Db-cAMP may exert its in vitro protective influence by activating protein kinase A and promoting endothelial cell membrane stability [22]. Following the observation of Shasby et al., who demonstrated the importance of actin filaments at the cell membrane in regulating cell permeability, Stelzer et al. [24] have shown that increased levels of cAMP increase endothelial apposition and have related this to increased levels of actin at the cell membrane. A further hypothesis has been suggested by Koyama et al. [25], who postulated that Db-cAMP attenuation of endotoxin lung injury in sheep was related to decreased prostaglandin production. In our model of Exo A-induced endothelial cell injury, the protective effects of Db-cAMP are not related to pressor effects. The mechanism of Db-cAMP protection in this model is dependent on intracellular mechanisms, as incubation of Db-cAMP with endothelial cells post-Exo A protects against exotoxin injury, and protection, therefore, is not dependent on binding extracellular exotoxin. The specific intracellular mechanism of protection in our model is not clear. Repletion of intracellular adenosine triphosphate (ATP) levels using cAMP analogues as a substrate for ATP formation, as suggested by Andreoli et al. [26], is a possibility. They have demonstrated that oxidant injury to endothelial cells can be reversed by augmenting intracellular cAMP levels [26]. In these studies, it appears that oxidant stress can be tolerated for a period of up to 90 min, after which cell "rescue" is impossible. It is interesting to note that high concentrations of Db-cAMP were toxic to the endothelial cells even in the absence of Exo A. This may be related to high levels of adenosine as a breakdown product of Db-cAMP, which have been demonstrated to cause cytotoxicity to endothelial cells in culture [27]. The possibility that after internalization Db-cAMP works as an intracellular secondary messenger in the form of cAMP, which then activates protein kinase A, has not been ruled out [22].

In summary, exposure of endothelial cells to Exo A need only be for a brief period before binding occurs, and the injury that follows is independent of extracellular Exo A. Furthermore, Db-cAMP can interrupt injury after binding of Exo A. This implies that the sequence of events involved in cell injury in this model is interruptable. However, our studies also show that Exo A-induced endothelial cell injury begins early, and, consequently, treatment of exotoxin-induced lung injury early enough to interrupt intracellular pathways will provide formidable problems in the clinical setting.

References


