

# Mitochondrial alterations induced by aspirin in rat hepatocytes expressing mitochondrially targeted green fluorescent protein (mtGFP)

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**Abstract** Mitochondria in primary living hepatocytes were visualized in cells transfected with a chimeric plasmid encoding for the green fluorescent protein (GFP) of *Aequorea victoria* engineered to be specifically targeted to mitochondria, as described recently (Rizzuto et al. (1995) *Curr. Biol.* 5, 635–642). The identification of the fluorescent organelles as authentic mitochondria was confirmed by double labeling with rhodamine 123. Acetylsalicylate treatment of hepatocytes induced in mitochondria typical morphological alterations closely analogous to the swelling promoted by acetylsalicylate in isolated mitochondria. Cyclosporin A, which in isolated mitochondria prevents the changes induced by acetylsalicylate, had no protective action but induced per se specific alterations in the morphology of mitochondria. Moreover, exposure of hepatocytes to cyclosporin A followed by acetylsalicylate caused the same mitochondrial changes induced by each of the two compounds separately. The structural alterations caused by acetylsalicylate were constantly associated with a decrease in mitochondrial urea synthesis and cell viability.

**Key words:** Acetylsalicylate; Cyclosporin A; GFP; Hepatocyte; Rat liver mitochondria

## 1. Introduction

Salicylates are known to induce in isolated liver mitochondria structural and functional alterations very similar to those found in Reye's syndrome [1–4]. Recently, it has been shown that the exposure of isolated rat mitochondria to 0.5 mM acetylsalicylate (ASA) induced all the alterations described for the mitochondrial permeability transition (MPT) process [5], i.e. large amplitude swelling, permeability to external sucrose, collapse of transmembrane potential and efflux of endogenous Mg<sup>2+</sup> and accumulated Ca<sup>2+</sup>. These alterations were fully prevented by either cyclosporin A (CsA) or Mg<sup>2+</sup> [6].

Despite their clear damaging action on isolated mitochondria salicylates were not able to induce all the alterations when tested in *in vivo* animal models [3]. This discrepancy raises the question of the sensitivity of intracellular mitochondria to the effect of salicylates when isolated cells are exposed to the drug.

The aim of the present work was to investigate the effect of ASA on mitochondrial morphology and function in primary

cultured hepatocytes. Mitochondria were visualized by: (i) transfecting the cells with a chimeric plasmid encoding for mitochondrially targeted GFP (mtGFP) [7]; and (ii) staining with the cationic dye rhodamine 123 accumulated in the matrix driven by membrane potential [8].

The results obtained with this procedure clearly show that in primary hepatocyte cultures exposed to 2–5 mM ASA, mitochondria undergo major and typical changes in structure and functionality, accounting for the lower viability and impaired function of the cells.

## 2. Materials and methods

### 2.1. Animals

Male rats of the Wistar strain, weighing 180–220 g, were maintained on standard laboratory food and water *ad libitum*. Experiments were started at 11:00 h. The animals were heparinized (500 U/100 g body wt) then anesthetized by the injection of ketamine intraperitoneally (16 mg/100 g body wt).

### 2.2. Hepatocyte culture and transfection

Liver parenchymal cells were isolated by collagenase perfusion according to Seglen [9], with minor modifications [10]. Yields of 3–4 × 10<sup>8</sup> cells/liver with 90–95% viability by trypan blue exclusion were routinely obtained.

After isolation the parenchymal cells were plated in flat-bottomed 6-well plates (Falcon) precoated with rat tail collagen, at a density of 6 × 10<sup>5</sup> cells/well in 2 ml of Williams' medium E containing 10 IU/ml penicillin, 10 µg/ml streptomycin, 0.005 µg/ml gentamycin, 0.02 units/ml insulin, and 10% heat-inactivated (56°C for 20 min) fetal calf serum. For microscopic observation, the cells were plated onto 24 mm diameter round coverslips precoated with rat tail collagen. After incubation for 3 h at 37°C in an atmosphere of 5% CO<sub>2</sub>, 95% air, the cultures were rinsed twice with prewarmed, HEPES (10 mM) buffered, Williams' medium E. Complete Williams' medium E was renewed, and the cells incubated overnight. Following overnight culture, hepatocytes were washed as above and placed in fresh complete Williams' medium E. For the transient expression of GFP the cells were transfected with mtGFP expression plasmid described by Rizzuto et al. [7] by the cationic-liposome (DOTAP) method, basically following the manufacturer's instructions. Briefly, DNA and the reagent were mixed in a ratio of 1:7 (w/w) and incubated for 15 min at room temperature. Finally, the mixture was added with complete Williams' medium E to the 3 h plated cells (4 µg per 35 mm well of plasmid DNA) and incubated at 37°C overnight.

### 2.3. Detection of mtGFP and rhodamine 123 fluorescence *in vivo*

mtGFP fluorescence was observed in living hepatocytes, 36–42 h after transfection with a Nikon RCM 8000 real-time confocal microscope. The coverslip with the mtGFP-transfected cells was placed in a chamber kept at 37°C by a thermostat (Medical System Corporation, Greenvale, NY) on the microscope stage. The sample was illuminated with an argon-ion laser using the 488 nm band. Williams' medium E buffered with 15 mM HEPES (pH 7.4) was used for the incubation.

Hepatocytes were incubated with 10 µM rhodamine 123 (dissolved in dimethyl sulfoxide) for 10 min at 37°C, washed several times with warm medium and then analyzed as described above.

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**Abbreviations:** ASA, acetylsalicylate; CsA, cyclosporin; MPT, membrane permeability transition; mtGFP, mitochondrially targeted green fluorescent protein

#### 2.4. Determination of urea synthesis and cell viability

Hepatocytes plated at a density of  $1 \times 10^6$  cells/well in 2 ml of complete Williams' medium E were exposed overnight to increasing concentrations of acetylsalicylate and/or cyclosporin and/or butocaine (all dissolved in dimethyl sulfoxide, the final concentration of which never exceed 0.5% v/v). In the morning, 10 mM ammonium chloride plus 5 mM ornithine, or 5 mM asparagine were added to induce urea synthesis (mitochondrial and cytosolic site, respectively). Samples of the incubation medium (0.15 ml) were collected at time 0 and after 6 h. 0.05 ml were used to assay lactate dehydrogenase (LDH) release and the remaining was deproteinized in ice-cold perchloric acid (6% final concentration) for urea determination [11]. At the end of the experiment Triton X-100 (0.5% final) was added to each well and a sample collected to determine the total LDH.

#### 2.5. Statistical analysis

Analysis of variance was used to evaluate differences among multiple treatment conditions. The Student-Neumann-Keuls test was then employed to test for statistical significance of any observed differences. Values of  $P > 0.05$  were considered to be not significant.

#### 2.6. Materials

Cyclosporin A (powder) was kindly provided by Sandoz (Berna, Switzerland). Transfection reagent (DOTAP) was obtained from Boehringer Mannheim. Collagenase (type IV), acetylsalicylic acid, rhodamine 123 and butocaine were purchased from Sigma. All other reagents were of the highest grade commercially obtainable.

### 3. Results

#### 3.1. Morphological modifications induced by ASA

2 days after transfection with the mtGFP cDNA, a proportion (5–10%) of the cells appeared strongly fluorescent, the label being restricted to a diffuse network of fine tubular structures throughout the cytosol, but excluding the nucleus (Fig. 1a).

This pattern of fluorescence is similar to that described in other cell types for mitochondria [7,8], though the density of these organelles is, as expected, much higher in hepatocytes than in other cells. The identification of the fluorescent organelles as mitochondria was confirmed by double labeling with rhodamine 123 (data not shown).

Overnight exposure (15–20 h) to concentrations of ASA above 2 mM induced dramatic alterations in mitochondrial morphology: transition from the 'spaghetti like' pattern of the untreated cell (Fig. 1a) to large cisternal or vacuolar structures (Fig. 1b,c). These changes resemble the mitochondrial swelling found in isolated mitochondria exposed to salicylates [2,3,6]. The possibility that these morphological alterations are due to an interaction between the recombinant GFP and ASA seems unlikely because the same changes in mitochondrial morphology were observed when untransfected ASA-treated cells were stained with rhodamine 123 (Fig. 1c). Unlike rhodamine, which undergoes rapid photobleaching, GFP fluorescence is very stable even under continuous illumination and allows a prolonged observation of the sample [7]. In addition, GFP fluorescence is unaffected by membrane potential, thus allowing the visualization of mitochondrial morphology also under conditions of reduced membrane potential.

Taking advantage of these properties of mtGFP, it was possible to follow the modifications of mitochondrial shape induced by ASA for up to 1 h in the same hepatocyte. Fig. 2 shows the changes of mitochondrial morphology in a single hepatocyte following 30 min exposure to 5 mM ASA (compare Fig. 2a with Fig. 2b). In this cell no further modifications occurred when the incubation with the drug was prolonged to 2 h.

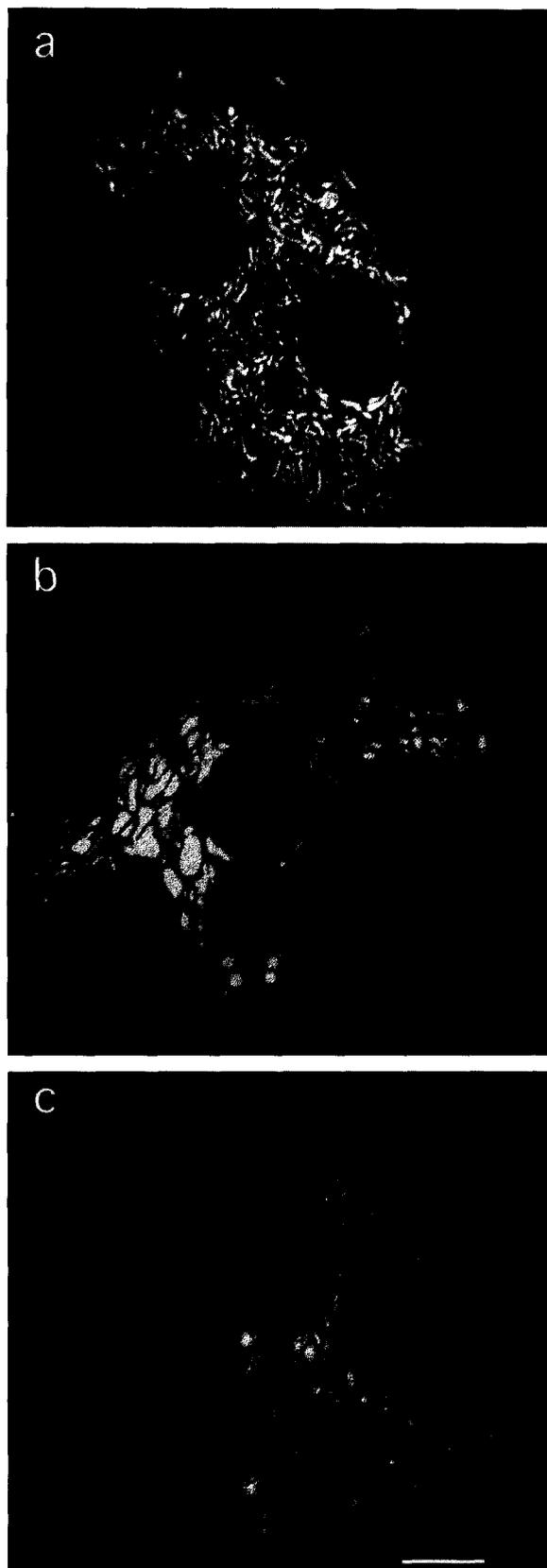


Fig. 1. Morphological alterations of mitochondria induced by overnight exposure to acetylsalicylate. Confocal images of (a) untreated hepatocytes, or (b,c) after overnight incubation with 3 mM ASA. Cells were transiently transfected with mtGFP (a,b), or stained with rhodamine 123 (c) as described in section 2. Scale bar = 10  $\mu$ m.

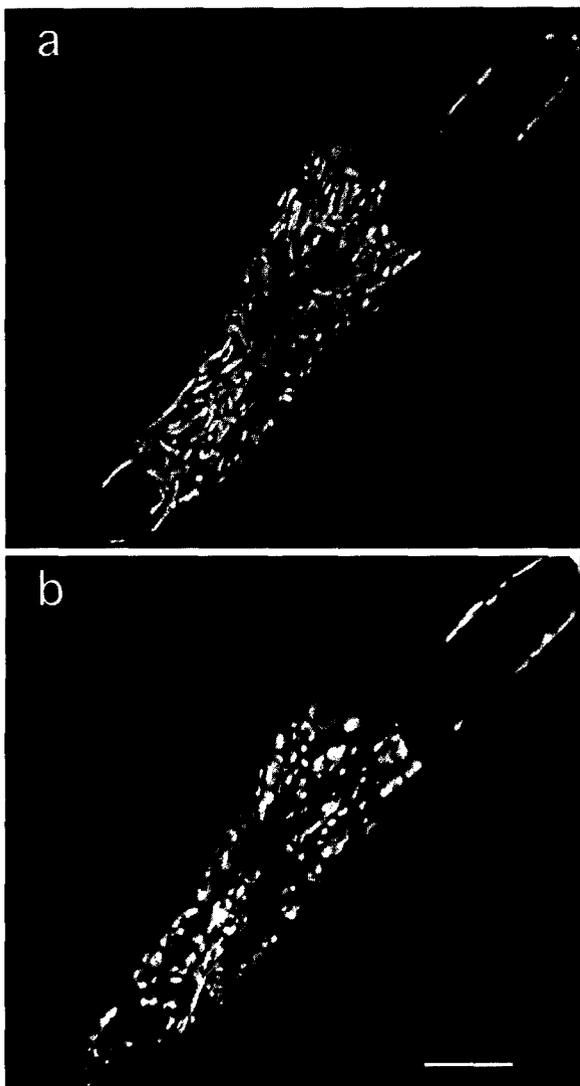


Fig. 2. Mitochondria alterations after short-term incubation with acetylsalicylate. Confocal images of the same hepatocyte transfected with mtGFP, (a) before and (b) after 30 min treatment with 5 mM acetylsalicylate, as described in section 2. Scale bar = 10  $\mu$ m.

Interestingly, during this short-term ASA treatment (approx. 2 h), only a minor proportion of the cell population (up to 5%) was affected; an overnight exposure to these ASA concentrations was required to induce these morphological alterations in most, if not all, hepatocytes. This indicates a gradual action of the drug suggesting a non uniform sensitivity of the cells to ASA.

ASA did not alter the cell volume, as inferred by: (i) the unchanged cell area in panels a and b of Fig. 2, (ii) the lack of modification of cell thickness, as estimated from the number of sections obtained by confocal scanning of the z-axis.

### 3.2. Cyclosporin A action

Since mitochondria seem to be a specific target of ASA, we assayed the possible protective action of CsA. While in isolated mitochondria CsA was shown to prevent the triggering of MPT by ASA [6], in transfected intact hepatocytes CsA did not modify the changes induced by ASA.

Conversely, when cultured hepatocytes were treated overnight with 5  $\mu$ M CsA alone, drastic alterations in mitochon-

drial morphology were noted. In particular, CsA induced shrinkage of mitochondria, with disruption of the typical morphology of healthy cells (Fig. 3a). These changes are radically different from those induced by ASA (Fig. 1b,c). Moreover, when the cells were pretreated for 1 h with 5  $\mu$ M CsA and successively exposed overnight to 3 mM ASA, mitochondria assumed a morphology consisting of a mixture of the modifications induced by each of the two compounds separately, either in different hepatocytes, or even in the same hepatocyte, as shown in Fig. 3b.

### 3.3. Cell viability and urea synthesis

The described morphological changes caused by ASA were constantly associated with a decrease of both cell viability and functional efficiency estimated by lactic dehydrogenase release and ammonia + ornithine-induced urea synthesis, respectively. As shown in Table 1, cell viability was dose-dependently reduced by ASA, in either the absence or presence of CsA, which, according to this parameter, failed to attenuate ASA toxicity. Likewise, CsA plus butocaine (40  $\mu$ M), a known inhibitor of phospholipase A which plays a protective role in the MPT [12], was without any appreciable effect on ASA-induced toxicity (data not shown). An inspection of the results reported in Table 1 reveals that CsA alone induced a small, but statistically significant, damage of the hepatocytes, increasing the LDH release from 8.26 to 15.53%. These data contrast with those of Pastorino et al. [13] who showed that, at the same concentration, the immunosuppressor was capable of preventing the death of cultured hepatocytes under hypoxic conditions. The damaging effect of ASA on intracellular mitochondria is also indicated by the data relative to urea synthesis from ammonium and ornithine reported in Table 2. These data, corrected for cell viability, show that ASA induced a statistically significant inhibition of urea production, in agreement with previous findings with freshly prepared hepatocytes [14]. CsA did not prevent this inhibition but, at variance with its action on hepatocyte viability, did not affect 'per se' the process of urea synthesis.

When cytosolic urea synthesis was sustained by 5 mM arginine the rate of urea production was not affected, indicating that only the part of urea synthesis occurring in mitochondria was impaired by ASA. It is noteworthy that the lowest concentration of ASA (1 mM) induced urea synthesis but not LDH release.

## 4. Discussion

Cationic dyes such as rhodamine 123 or JC-1 [15] have been

Table 1  
Hepatocyte viability after overnight exposure to acetylsalicylate and cyclosporin A

Additions	Lactate dehydrogenase release (% of total)	
	+CsA 5 $\mu$ M	
None	8.26 $\pm$ 3.15	15.53 $\pm$ 3.58 <sup>a</sup>
ASA 1 mM	12.89 $\pm$ 2.66	17.18 $\pm$ 5.05 <sup>b</sup>
ASA 3 mM	19.16 $\pm$ 3.60 <sup>b</sup>	23.71 $\pm$ 4.02 <sup>b</sup>
ASA 5 mM	29.16 $\pm$ 1.32 <sup>b</sup>	31.64 $\pm$ 0.60 <sup>b</sup>

Experimental conditions were as described in section 2; cyclosporin A was added 1 h before acetylsalicylate. Values are means  $\pm$  S.D. of 3–4 experiments, each one performed in triplicate.

<sup>a</sup>  $P < 0.01$ ; <sup>b</sup>  $P < 0.001$  vs. no addition.

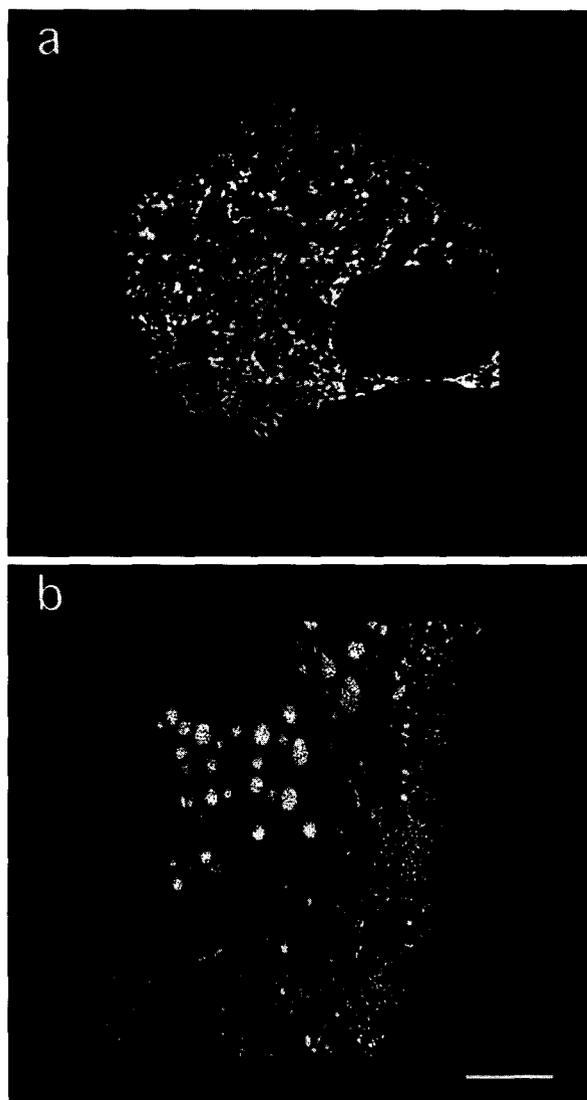


Fig. 3. Effect of cyclosporin A on mitochondrial shape. Confocal images of hepatocytes transfected with mtGFP, (a) after overnight treatment with 5  $\mu$ M cyclosporin A or (b) with 5  $\mu$ M cyclosporin A plus 3 mM ASA, as described in section 2. Scale bar = 10  $\mu$ m.

widely employed to monitor the morphology of mitochondria in living cells, though their use is not devoid of problems, such as toxicity and rapid photobleaching [7,16,17]. In addition, given that the staining is dependent on membrane potential, these dyes are not ideal to study the morphological changes of mitochondria under conditions, e.g. anoxia or treatment with mitochondrial poisons, which specifically affect mitochondrial membrane potential. In this study we have taken advantage of the new methodology described by Rizzuto et al. which consists in the transfection of cells with the cDNA coding for a mitochondrial targeted GFP. The consistency of the data obtained in cells transfected with mtGFP and controls stained with rhodamine 123 not only allows one to exclude artefacts due to the specific technique, but is methodologically particularly relevant because it confirms that expression of mtGFP is non-toxic even in a rather sensitive cell type such as hepatocytes in primary culture.

By the use of mtGFP, it has been possible to demonstrate that exposure of cells to ASA concentrations in the range

found in children with severe salicylate intoxication [18] alters the morphology of mitochondria rapidly and drastically 'in situ'. These alterations consist in swelling and rounding of the organelles, reminiscent of the morphological changes observed at the electron microscope level in isolated mitochondria exposed to ASA and  $\text{Ca}^{2+}$  [2]. The dramatic alteration caused by ASA (up to 3 mM) on mitochondrial morphology contrasts with the relatively minor effects of the drug on cell viability. These observations, together with the marked reduction in mitochondrial urea synthesis and the lack of effect of ASA on cell volume and cytosolic urea production, suggest that mitochondrial alterations could be a significant component of ASA toxicity, rather than the consequence of a generalized modification of cellular homeostasis. As to the mechanism of mitochondrial ASA toxicity, a major difference exists between the results obtained in vitro [6] and those reported here for intact cells. In the former case, ASA toxicity not only required a concomitant loading with high  $\text{Ca}^{2+}$  but, more importantly, was effectively inhibited by CsA, suggesting the involvement of the permeability transition pore [6]. In intact cells, on the contrary, CsA had no protective effect on either the morphological alterations or the metabolic impairment induced by ASA. Furthermore, though changes in  $\text{Ca}^{2+}$  homeostasis in ASA-treated cells were not studied directly, no plasma membrane blebbing, a classical morphological characteristic of impaired cellular  $\text{Ca}^{2+}$  homeostasis, was observed in cells undergoing changes in mitochondrial morphology. The lack of membrane blebbing and of a protective effect by CsA thus suggests that the morphological and metabolic changes induced by ASA in living cells are not due to an effect of the salicylate on the MPT. At present, however, it cannot be excluded that, due to the limited permeability of the plasma membrane to CsA and the presence of non-mitochondrial CsA binding proteins (cyclophilins) [19], the drug never reaches, in the cytosol, the effective concentrations needed to affect the MPT. Last but not least, CsA presented, on its own, a small but significant toxicity towards cultured hepatocytes and caused characteristic alterations in mitochondrial morphology. To our knowledge, these changes in mitochondrial shape have not been described previously and their functional significance remains undetermined and will require further investigation.

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Table 2  
Effect of acetylsalicylate and cyclosporin A on the rate of urea synthesis of primary cultured hepatocytes

Additions	Urea production (nmol/h per $10^6$ living cells)	
	+ CsA 5 $\mu$ M	
None	550.56 $\pm$ 52.03	508.70 $\pm$ 14.97
ASA 1 mM	472.70 $\pm$ 27.42 <sup>a</sup>	482.91 $\pm$ 23.54 <sup>a</sup>
ASA 3 mM	379.11 $\pm$ 27.80 <sup>b</sup>	404.58 $\pm$ 19.84 <sup>b</sup>
ASA 5 mM	380.05 $\pm$ 25.10 <sup>b</sup>	400.80 $\pm$ 20.12 <sup>b</sup>

Experimental conditions were as described in section 2; cyclosporin A was added 1 h before acetylsalicylate. Values are means  $\pm$  S.D. of 3–4 experiments, each one performed in triplicate.

<sup>a</sup> $P < 0.05$ ; <sup>b</sup> $P < 0.001$  vs. no addition.

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