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# Requirement of nitric oxide and calcium mobilization for the induction of apoptosis in adrenal vascular endothelial cells

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**Abstract Exposure of adrenal vascular endothelial cells (AVEC) to pharmacological nitric oxide (NO) donors, pro-inflammatory cytokines or lipopolysaccharide was unable to induce apoptosis as occurred when macrophages were treated under identical experimental conditions. However, when the intracellular $Ca^{2+}$ concentration increased, AVEC displayed apoptotic features upon exposure to NO. This apoptosis was confirmed by the release of oligonucleosomes to the cytosol and by the characteristic DNA laddering observed after electrophoresis in agarose gels. $Ca^{2+}$-mobilizing agents and interleukin-1β (IL-1β) also elicited an apoptotic response in these cells through a mechanism that required NO synthesis. The ability of NO and intracellular $Ca^{2+}$ to promote apoptosis was dependent on the number of passages of the cells in culture, suggesting the loss of protective factors in the course of ex vivo cell culture. Because AVEC exhibit an important capacity to increase the intracellular $Ca^{2+}$ concentration in response to a wide array of agonists, this condition might affect the integrity of the vascular system under pathological circumstances such as those prevailing in the course of septic shock.**



*Key words:* Apoptosis; Endothelial cell; Nitric oxide; Calcium agonist

## 1. Introduction

The ability of nitric oxide (NO) to induce apoptosis has been well defined in different types of cells including macrophages, several subsets of lymphocytes, tumor cells and β-pancreatic cells [1–3]. This NO-dependent apoptosis requires the release of high amounts of NO, a process usually accomplished through the expression of the high output type II NO synthase (NOS2) [4]. The activity of NOS2 is mainly controlled at the transcriptional level and exhibits a cell-specific pattern of expression. Pro-inflammatory cytokines such as Tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interferon-γ (IFN-γ), or bacterial cell wall products such as lipopolysaccharide (LPS) are among the most efficient factors to trigger NOS2 expression [4,5].

Under physiological conditions endothelial cells contain only the $Ca^{2+}$/calmodulin-dependent NOS3, which catalyzes the synthesis of moderate amounts of NO in response to increases in cytosolic $Ca^{2+}$ [6]. However, in the course of septic shock, a process in which bacteria reach the systemic circulation and an intense inflammatory reaction occurs [7,8], vascular endothelial cells express NOS2 [9]. Cultured vascular endothelial cells down-regulate NOS3 and express NOS2 upon LPS challenge, which results in the synthesis of important amounts of NO [10,11]. According to these observations, and in view of the ability of NO to elicit rapid apoptotic cell death, we decided to investigate whether NO might induce apoptosis in adrenal vascular endothelial cells (AVEC) and in this way contribute to the alterations observed in the vascular system under septic shock conditions. Our results show that cultured AVEC are very resistant to suffer apoptosis in response to NO challenge. However, a marked potentiation of apoptosis is observed when a simultaneous increase in intracellular calcium and NO synthesis occurs.

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*Abbreviations:* AVEC, vascular endothelial cell(s); LPS, lipopolysaccharide; NOS2, cytokine-inducible $Ca^{2+}$-inducible nitric oxide synthase; TNF-α, tumour necrosis factor-α; SIN-1, 3-morpholino-sydnonimine; SIN-10, molsidomine; GSNO, *S*-nitrosoglutathion; SNAP, *S*-nitroso-*N*-acetyl-penicillamine

## 2. Materials and methods

### 2.1. Chemicals

LPS, cytokines, and NO donors were from Sigma. Other reagents were from Boehringer or Merck.

### 2.2. Preparation and characterization of AVEC

AVEC were prepared from collagenized bovine adrenal medulla after differential plating [12]. Briefly, the suspension of adrenomedullary cells was seeded in 75 $cm^2$ culture flasks at $30\times10^6$ cells per 30 ml of DMEM supplemented with 10% FCS, 50 μg/ml penicillin, 50 μg/ml streptomycin, 100 μg/ml kanamycin, and 3 μg/ml of amphotericin. After a settling period of 3 h at 37°C in 5% $CO_2$ and 95% air, the unattached cells were aspirated and the cell layer was extensively washed with the medium described above. When cells reached confluency (≈ 1 week), they were trypsinized and subcultured at $6\times10^4$ cells/$cm^2$. Except otherwise stated, cultures of 2nd to 3rd passage were used. The cells were characterized by staining with a labelled antibody against Factor VIII-related antigen (> 80% positive cells) and by immunocytochemistry with anti-bovine endothelial NOS (> 92% positive cells) using an antibody donated by Dr. S. Lamas (Instituto de Investigaciones Biológicas, Madrid) [6,13].

### 2.3. Culture of RAW 264.7 cells

RAW 264.7 murine macrophages were obtained from the ATCC. To standardize the culture media macrophages were maintained in DMEM medium supplemented with 2 mM glutamine, 10% FCS and antibiotics.

### 2.4. Synthesis of GSNO and determination of $NO_x^-$ concentration

GSNO was synthesized from glutathione and $NaNO_2$ following a previous protocol [14]. GSNO was precipitated with acetone and purified after extensive washing with diethylether. After vacuum drying, an aliquot was characterized by UV spectroscopy [14]. NO release was measured by the accumulation of nitrite and nitrate in the culture medium as previously described using Griess reagent [15].

### 2.5. Measurement of LDH activity

To evaluate the plasma membrane integrity the release of LDH to the extracellular medium was measured after centrifugation of the culture medium at $15000\times g$ for 10 min. LDH activity was assayed in the presence of 0.5 mM pyruvate and 0.15 mM NADH [16].


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*2.6. Measurement of intracellular calcium mobilization*

AVEC were seeded over cover-slips and loaded with Fura-2 as described [12]. Intracellular calcium mobilization was followed by the changes in the fluorescence at 510 nm after dual excitation at 340 and 380 nm [12].

*2.7. Analysis of DNA fragmentation*

Internucleosomal DNA fragmentation was assessed by distinct independent methods including agarose gel electrophoresis analysis of fragmented DNA, and a cell death ELISA kit (Boehringer) based on the detection of mono- and oligonucleosomes in the cytosol [14,15]. The dishes ($1–2\times10^6$ cells) were washed twice with ice-cold PBS and lysis was accomplished with 1 ml of 20 mM EDTA, 0.5% Triton X-100, 5 mM Tris-HCl, pH 8.0. After incubation of the dishes for 15 min at 4°C with gentle shaking, the cell extract was treated for 1 h at 55°C with 0.3 mg/ml of proteinase K. After two extractions with phenol/chloroform, the DNA was resuspended and analyzed in a 2% agarose gel after staining with 0.5 μg/ml of ethidium bromide. Alternatively, aliquots of lysed cells were centrifuged at $500\times g$ for 10 min to remove the nuclei and the content of mono- and oligonucleosomes in the cytosol was detected using a sandwich-enzyme-immunoassay with anti-histone and anti-DNA-peroxidase antibodies. The relative degree of apoptosis was determined quantitatively by measuring the peroxidase activity at 405 nm and calculating the ratio between the activity of a sample at the indicated time of sampling and the corresponding activity at $t=0$ h (enrichment factor). The average $A_{405}$ of samples from control cells was 0.11 absorbance units per 15 min of reaction.

*2.8. Data analysis*

Statistical differences ($P<0.05$) between mean values were determined by 1-way analysis of the variance (ANOVA) followed by Student's $t$ test. $P<0.05$ was considered significant.

## 3. Results

*3.1. NO donors and LPS fail to induce apoptosis in AVEC*

To determine the ability of NO to induce DNA degradation of bovine AVEC, SIN-1, a chemical NO donor, was added to the culture medium and the integrity of the DNA was analyzed by electrophoresis in an agarose gel. As Fig. 1A shows, SIN-1 assayed at 0.5 mM was unable to significantly induce DNA degradation in these cells. To ensure that the absence of NO-dependent DNA degradation was not due to a restricted permeability of the cells to SIN-1, intracellular NO synthesis was accomplished after expression of NOS2 by cells challenged with LPS, and following the release of nitrite to the culture medium. However, intracellular NO synthesis was unable to elicit apoptosis in these cells. Fig. 1A also shows the apoptotic profile of a macrophage cell line (RAW 264.7 cells) treated under identical conditions as bovine AVEC. In agreement with a previous work, both NO donors and LPS treatment were self-sufficient conditions to promote apoptosis in macrophages [1,15]. To obtain a more quantitative analysis of the DNA degradation, the release of oligonucleosomes from the nucleus towards the cytosol was followed using a two-antibody (anti-histone and anti-DNA) sandwich ELISA. As Fig. 1B shows, a marked DNA degradation was observed after treatment of macrophages for 24 h with SIN-1, whereas this behavior was absent in AVEC over the 48 h period of observation.

Since chemical NO donors might induce different effects depending on the rate of decomposition and the interaction of the residual moieties of the donor molecule with the metabolism of the target cells (i.e. synthesis of peroxinitrites from SIN-1) experiments were undertaken using several NO donors. As Fig. 2 shows, among the NO donors assayed, only minimal but statistically significant apoptosis was observed when GSNO was used. When the effect of NO donors was combined with LPS treatment of AVEC, again only GSNO was effective (Fig. 2).

*3.2. Synergism of NO donors, IL-1β and $Ca^{2+}$-mobilizing agonists to induce apoptosis in AVEC*

Since different pathways might convey in promoting apoptosis, we investigated the effect of pro-inflammatory cytokines, $Ca^{2+}$-mobilizing agonists and NO donors on the induction of apoptotic cell death in AVEC. As Fig. 3A shows, IL-1β induced a modest apoptosis in these cells, a response that was potentiated in the presence of GSNO. However, LPS and TNF-α were ineffective and failed to synergize with IL-1β. Moreover, in the presence of a saturant concentration of IL-1β (20 nM; assays not shown) GSNO enhanced the apoptotic response, which suggests that IL-1β and NO are activating complementary pathways controlling apoptotic death (Fig. 3B). However, concentrations of GSNO higher than 0.5 mM resulted in a marked toxicity as reflected by the release of LDH activity to the medium (Fig. 3B).

AVEC well respond to endothelial $Ca^{2+}$-mobilizing agonists [17], among them $P_{2U}$ agonists [12], and increases of intracellular $Ca^{2+}$ have been related to apoptotic death [18]. For this reason, we have analyzed in cells treated with NO donors the effect of $Ca^{2+}$ mobilization on apoptosis. As Fig. 4A shows, ATP, ATPγS and UTP, but not ADP, promoted an important $Ca^{2+}$ increase. Although acting alone these nucleotides did not affect apoptosis, when cells were stimulated in the presence of GSNO, an enhanced accumulation of oligonucleosomal DNA in the cytosol was observed (Fig. 4B). ADP, which exerted minimal effects on $Ca^{2+}$ mobilization, was ineffective promoting apoptosis. Moreover, the simulta-

Table 1
Effect of cell passage on the apoptosis elicited by IL-1β, $Ca^{2+}$-mobilizing agonists and NO donors in AVEC

| Treatment | Nucleosomal enrichment factor | | |
|---|---|---|---|
| | 4th passage | 6th passage | 8th passage |
| None | 1.0 ± 0.1 | 1.0 ± 0.2 | 1.1 ± 0.3 |
| GSNO (0.5 mM) | 1.8 ± 0.2* | 2.5 ± 0.3** | 3.7 ± 0.2** |
| IL-1β (20 nM) | 2.1 ± 0.3* | 3.4 ± 0.3** | 3.5 ± 0.3** |
| ATP (200 μM) | 1.7 ± 0.2* | 2.1 ± 0.2* | 2.4 ± 0.3* |
| A23187 (200 nM) | 1.4 ± 0.2 | 2.0 ± 0.3* | 2.9 ± 0.3** |
| GSNO+IL-1β+ATP | 3.8 ± 0.4** | 4.5 ± 0.5** | 7.2 ± 0.4** |
| GSNO+IL-1β+A23187 | 4.0 ± 0.3** | 4.7 ± 0.5** | 8.4 ± 0.7** |

Cells of 4th, 6th or 8th passage were challenged with the indicated stimuli and the release of oligonucleosomes to the cytosol was measured after 24 h of treatment. Results show the mean ± SEM of three experiments.
*$P<0.05$; **$P<0.001$ compared to untreated cells.

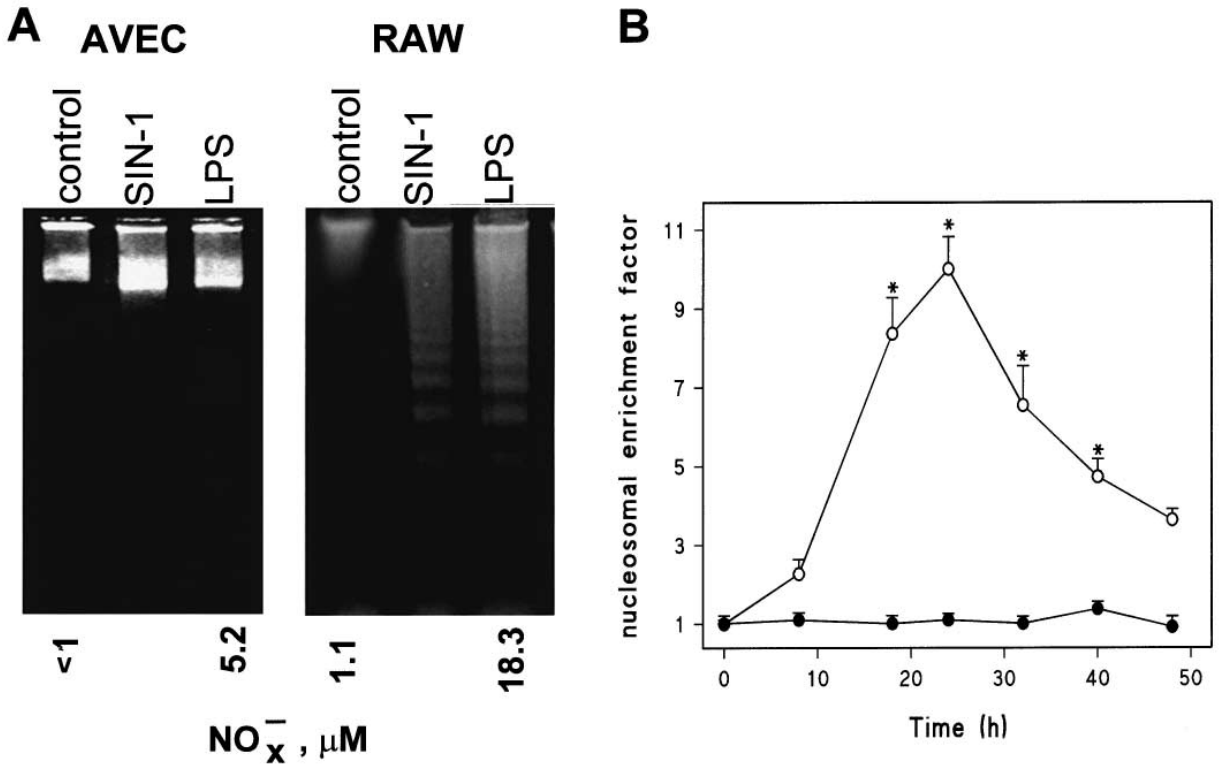


Fig. 1. NO and LPS fail to induce apoptotic cell death in cultured AVEC. Cells were challenged with 0.5 mM SIN-1 or 1 μg/ml of LPS and after 24 h of culture the DNA was purified and analyzed in an agarose gel. The same experiment was performed using RAW 264.7 cells. The synthesis of NO from cells treated with LPS was measured as the amount of nitrite released to the medium over a period of 24 h (A). Alternatively, AVEC (●, 2nd passage) and RAW 264.7 macrophages (○) were stimulated with 0.5 mM SIN-1 and the amount of oligonucleosomes present in the cytosol was measured at the indicated times (B). Results show a representative experiment out of three (A) or the mean ± SEM of three experiments assayed per duplicate. $^*P < 0.001$ with respect to cells at $t = 0$ h (B).

neous presence of ATPγS, IL-1β and GSNO potentiated the apoptosis of AVEC. In addition to the measurement of oligonucleosomes in to the cytosol the integrity of the DNA was examined in agarose gels. As Fig. 5 shows, treatment of AVEC with the $Ca^{2+}$ ionophore A23187 and IL-1β or GSNO, potentiated the DNA degradation, an effect reinforced when the three stimuli were simultaneously present. These results were in agreement with the data obtained measuring oligonucleosomes in the cytosol.

Finally, in the course of continuous cell passages, an increased sensitivity of AVEC to display apoptosis in response to NO donors, $Ca^{2+}$ agonists and IL-1β was observed. As shown in Table 1, treatment of AVEC of 4th, 6th or 8th passage with GSNO, IL-1β, and to a less extent $Ca^{2+}$-mobilizing agents, resulted in enhancement of apoptosis. These results indicate that the sensitivity of cells to induce apoptosis in response to extracellular stimuli increases in parallel with the number of cell passages.

## 4. Discussion

Our results show that primary cultures of bovine AVEC were extremely resistant to apoptosis after exposure to large amounts of NO. This observation was in contrast to the high sensitivity of RAW 264.7 macrophages and other cells to display NO-dependent apoptosis under similar experimental conditions [1,14,15]. Moreover, treatment of various cell types with SIN-1 constitutes a sufficient condition to elicit apoptosis [1,15,19]. NO by itself is a weak oxidant, but after reaction with oxygen superoxide is converted into peroxinitrite, an even more reactive species [20,21]. In this regard, the effectiveness of SIN-1 in promoting apoptosis has been related to the synthesis of peroxinitrite in the course of NO release [20,21], and for this reason it was the NO donor of choice to perform the experiments in AVEC. However, according to our results,

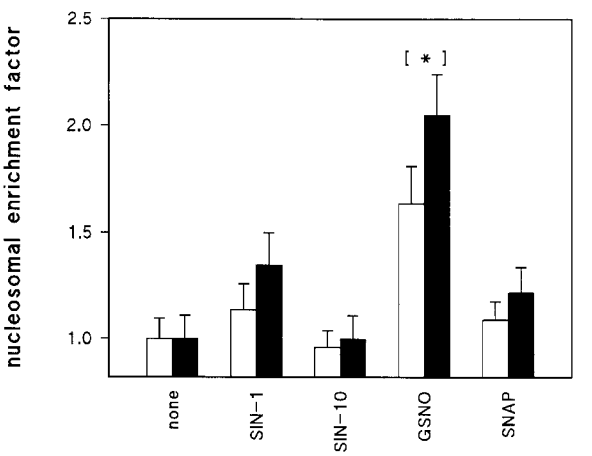


Fig. 2. Effect of several NO donors and LPS on the release of oligonucleosomes to the cytosol. AVEC were stimulated for 24 h with the indicated NO donors (assayed at 0.5 mM) in the absence (open bars) or presence of 1 μg/ml of LPS (solid bars). Apoptosis was followed by the release of oligonucleosomes to the cytosol. Results show the mean ± SEM of three experiments. $^*P < 0.01$ with respect to the none (control) condition.

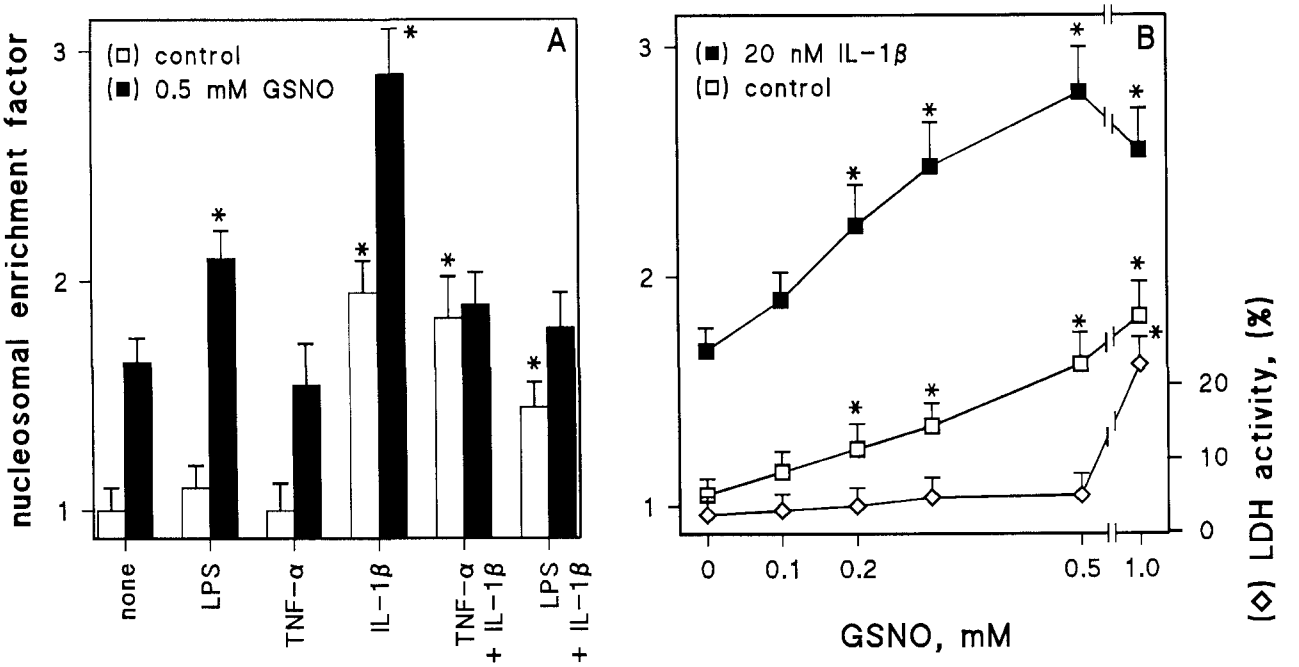


Fig. 3. Effect of extracellular stimuli on apoptosis of AVEC. Cells (2nd passage) were stimulated for 24 h with 1 μg/ml of LPS, 20 ng/ml of TNF-α and 20 nM IL-1β, in the absence (open bars) or presence (solid bars) of 0.5 mM GSNO. The release of oligonucleosomal moieties to the cytosol was measured using an anti-histone and anti-DNA sandwich ELISA (A). Also, AVEC were stimulated for 24 h with IL-1β and increasing concentrations of GSNO and the amount of oligonucleosomes present in the cytosol was measured. The release of LDH activity to the medium was followed as a marker of necrotic death (B). LDH activity was expressed as percentage of the extractable activity when the culture was treated with 0.5% Nonidet P-40 and homogenized. Results show the mean ± SEM of the enrichment of the cytosol in oligonucleosomes of three experiments. $^*P < 0.05$ compared to untreated cells.

GSNO, but not SIN-1, was the unique NO donor among those assayed which exhibited a significant effect in promoting apoptosis of AVEC. The molecular basis of this specificity for GSNO remain unclear, but the by-products released by the glutathione-derived moiety could contribute to these effects. Indeed, using endothelial cells derived from human umbilical vein, Lin et al. [20] have recently observed a special refractory tendency of these cells to display apoptosis when treated with peroxinitrite. Moreover, in addition to the absence of an apoptotic response to chemical NO donors, treatment of AVEC with LPS, a self-sufficient condition triggering apoptosis in primary cultures of macrophages regardless of NO synthesis [15,19], did not affect cell viability, suggesting the existence of pathways which improve the viability of AVEC, at least in culture. Indeed, a certain protection by low doses of NO against apoptosis has been reported [22].

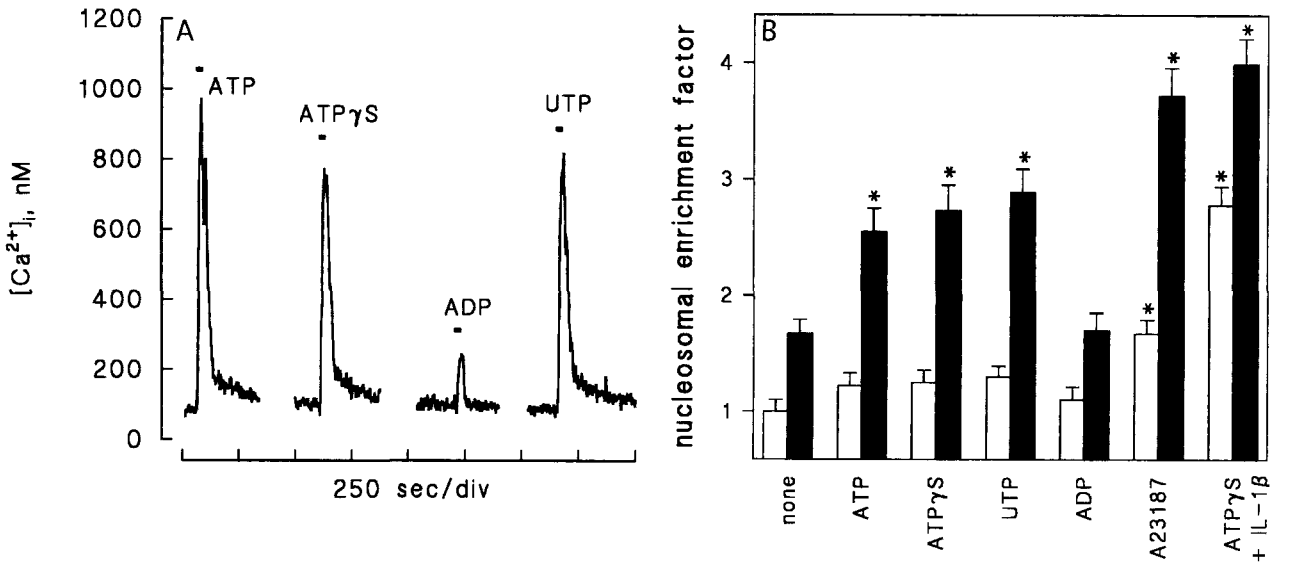


Fig. 4. $Ca^{2+}$ mobilization potentiates the apoptosis induced by GSNO. $Ca^{2+}$ mobilization was measured in Fura-2 loaded AVEC stimulated with 200 μM ATP, ATPγS, UTP or ADP (A). The release of oligonucleosomes to the cytosol was measured after 24 h of incubation in the absence (open bars) or presence (solid bars) of 0.5 mM GSNO. Results show the mean ± SEM of four experiments. $^*P < 0.05$ compared to non-stimulated cells.

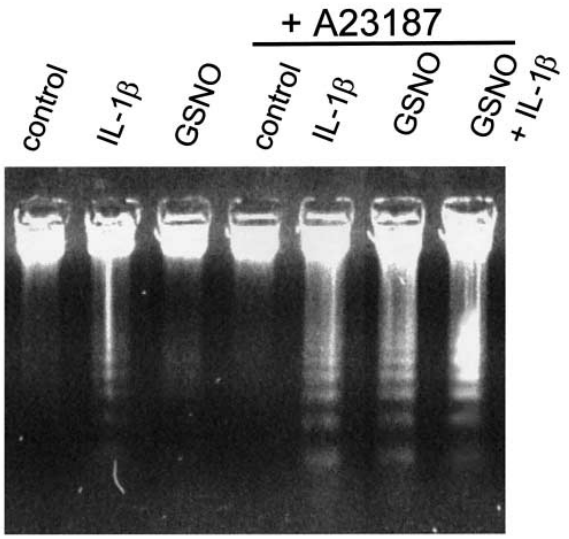


Fig. 5. Synergism of $Ca^{2+}$ mobilization, IL-1β and GSNO in the induction of DNA laddering in AVEC. Cells (2nd passage) were incubated for 18 h with 2 nM of IL-1β, 0.5 mM GSNO and 200 nM A23187, and the DNA was isolated and size-fractionated in an agarose gel.

Different pathways involved in the development of an apoptotic response have been characterized in several cell types, including cells of the neural and immune systems [22,23]. The view emerging from these studies is the existence of a wide multiplicity of pathways that either promote or protect from apoptosis depending on the cell type and on specific factors such as the status in the cell cycle, or the number of passages in culture [22–24]. Our results clearly point out the existence of concerted mechanisms which finally promote apoptosis in AVEC. Some NO donors (GSNO), the pro-inflammatory cytokine IL-1β or $Ca^{2+}$-mobilizing agents, induce a moderate apoptosis. Interestingly, TNF-α which usually is more efficient than IL-1β in promoting apoptosis in many cell types [25], had negligible effects under our experimental conditions. Several possibilities could be envisaged to explain this anomalous behaviour, ranging from a reduced number of TNF-α receptors to a low effectiveness because of the use of a murine source of cytokine. Simultaneous treatment of cells with IL-1β and GSNO revealed the existence of a synergistic effect on apoptosis at subsaturant concentrations of cytokine. In the same way, $Ca^{2+}$-mobilizing agonists, as well as the $Ca^{2+}$ ionophore A23187, proved very effective in promoting apoptosis of AVEC challenged with IL-1β and NO donors.

Cultured bovine AVEC well respond through the $P_2$ purinoceptor [12]. Consequently, the effectiveness of the $Ca^{2+}$-mobilizing agonists to potentiate apoptosis exhibited a parallelism with the ability of these stimuli to increase the intracellular $Ca^{2+}$ concentration [12], and the absence of response to ADP supports the engagement of a $P_{2Y}$-operated mechanism.

Taken together, the results reported in this paper suggest that AVEC express constitutively high levels of factors which efficiently protect them from apoptosis under quite aggressive conditions, including peroxinitrite synthesis. These factors, presumably proteins which improve cell viability, decrease in the course of cell passages rendering cells more sensitive to the action of agents that promote apoptosis. Moreover, the resistance of AVEC to suffer apoptosis appears to be a general property of these cells in view of the results obtained with human endothelial cells [21]. In this regard, the characterization of the pathways which protect against apoptosis may contribute to a better understanding of general mechanisms that prevent apoptosis as well as the relevance of this fact in the physiopathology of the vascular system. In this way, the high resistance of endothelial cells to suffer apoptosis contributes to attenuate vascular dysfunctions under pathological circumstances, as occurs for example under septic shock conditions.

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