# Stimulatory effect of ouabain on VCAM-1 and iNOS expression in murine endothelial cells: involvement of NF-κB

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**Abstract** **Endothelial cells play a pivotal role in the development of atherosclerosis. An 'activated' phenotype of these cells is manifested by signal transduction-dependent expression of genes encoding cytokines, pro- and anticoagulant factors, and cell adhesion molecules. In the current study we examined the effect of ouabain, an inhibitor of $Na^+/K^+$-ATPase, on the process of endothelial cell activation. We demonstrated that ouabain was able to stimulate VCAM-1 expression and potentiate the effect of IFN-γ on this process. Moreover, ouabain provided a complementary signal for either TNF or IFN-γ in inducing iNOS expression. Our data also show, for the first time, that inhibition of $Na^+/K^+$-ATPase led to activation of the transcription factor, NF-κB, which may provide an explanation for the effects of ouabain on endothelial cells.**

*Key words:* Ouabain; $Na^+/K^+$-ATPase; VCAM-1; iNOS; NF-κB

## 1. Introduction

At sites of inflammation, endothelial cells undergo specific alterations in their morphology, metabolism and functions. This process, called endothelial activation, is induced mostly by bacterial products and cytokines released by cells of the immune system. Changes in expression of genes during this process involve a complex program of intracellular signal transduction processes and transcription events. The common feature of these genes is the presence of the κB DNA regulatory element interacting with the transcription factor NF-κB (for review, see [1]). Activation of this factor occurs in cells exposed to TNF, IL-1, LPS, viral infections, activators of PKC and reactive oxygen intermediates (ROI) [2]. Both, vascular cell adhesion molecule-1 (VCAM-1) and inducible nitric oxide synthase (iNOS) genes contain in their promoter regions sequences that are recognized by NF-κB [3,4].

Because of the complexity of transcriptional regulation, the activation of cells depends not only on the repertoire of cytokines but also on the presence of other mediators as well as environmental conditions which may have inhibitory, permissive or intensifying effects for the action of cytokines [5,6]. $Na^+/K^+$-ATPase which catalyses the active transport of $Na^+$ and $K^+$ across the plasma membrane, plays a fundamental role in maintaining cell homeostasis (for review, see [7]). Modulation of its activity directly influences membrane potential, cell volume, active ion transport, cellular uptake of nutrients but may also indirectly affect many other processes in various cell types. The activity of $Na^+/K^+$-ATPase is under multifactorial regulation by hormones, neurotransmitters, and ions. Recently, IL-1 and endothelin have been found to cause inhibition of $Na^+/K^+$-ATPase activity [8,9]. Ouabain (strophantin G), a cardiac glycoside, specifically binds to the extracellular part of $Na^+/K^+$-ATPase and inhibits its activity [10] thus allowing to study the involvement of this pump in various processes. Recently, a potent inhibitor of $Na^+/K^+$-ATPase has been isolated from human plasma and found to be indistinguishable from ouabain [11]. It has been proposed that endogenous ouabain originates from the adrenal gland [12]. Utilization of ouabain allowed to demonstrate that inhibition of $Na^+/K^+$-ATPase not only affects basic metabolic processes but may also influence gene expression. It has been shown that ouabain stimulates TNF gene expression in murine macrophages [13] and the expression of GM-CSF in human embryonal fibroblasts [14].

Although much attention has been focused on ouabain as a potent regulator of vascular tone [15], little is known about its effect on endothelial cells or the possibility of its involvement in cell activation during inflammatory processes. The aim of our study was to examine the effect of ouabain on endothelial cell activation and especially on stimulation of the expression of VCAM-1 and iNOS.

## 2. Materials and methods

### 2.1. Reagents

Recombinant human TNF was purchased from RαD Systems (Minneapolis, MN). Recombinant murine IFN-γ, DMEM, RPMI 1640, FCS were obtained from Life Technologies Ltd. (Paisley, UK). Ouabain (endotoxin tested), lipopolisaccharide (from *E. coli*, serotype 055:B5) and E-Toxate kit (Limulus lysate) were purchased from Sigma Chemical Co., (St. Louis, MO). All radiolabeled compounds were purchased from Amersham Life Science (Little Chalfont, UK). Unless indicated differently all remaining reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

### 2.2. Maintenance of cells

Murine brain microvascular endothelial cells (MME) were a gift from Dr. R. Auerbach (Madison, WI). They were grown in medium consisting of DMEM, 20% FCS, endothelial cell growth supplement (30 μg/ml), 2 mM glutamine and penicillin (100 U/ml) and streptomycin (100 μg/ml). Rat peritoneal macrophages were harvested from the peritoneal cavity of adult rats 5 days after intraperitoneal injection of 10 ml of thioglycollate broth and cultured in RPMI supplemented with 10% FCS and antibiotics. P388/D1, murine macrophage-like cell line

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*Abbreviations:* PBS, phosphate-buffered saline; FCS, fetal calf serum; IFN-γ, interferon-γ; IL-1, interleukin 1; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; MME, murine microvascular endothelial cells; $Na^+/K^+$-ATPase, $Na^+$ and $K^+$-dependent adenosine triphosphatase; NF-κB, nuclear factor κB; TNF, tumor necrosis factor-α; VCAM-1, vascular cell adhesion molecule-1.


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were cultured in RPMI 1640 supplemented with 10% FCS and antibiotics. P815, murine mastocytoma cells were maintained in DBA/2J mice (Jackson Laboratory, Bar Harbor, ME). The cells were passaged every 5 to 6 days by intraperitoneal transfer of 0.1 ml ascites fluid.

*2.3. Adhesion assay*

Adhesion assay was performed as described previously [16]. P815 cells were labeled with $^{51}Cr$ and coincubated for 30 min with MME monolayers pretreated for 24 h with the indicated factors. The monolayers were then washed 3 times with PBS to remove nonadherent cells. The content of each well was solubilized by adding 0.2 ml of 0.5 M NaOH and the level of radioactivity was determined in a Packard gamma counter. The percentage of cells that adhered was calculated as:

$$\% \text{ of binding} = \frac{\text{labeled cells bound to monolayer}}{\text{labeled cells added to monolayer}} \times 100.$$

*2.4. Flow cytometric analysis*

MME monolayers, cultured in 6-well plates, were incubated for 24 h in RPMI 1640 supplemented with 2% FCS in the presence of factors as indicated. The cells were released from the plates with trypsin, washed twice with RPMI 1640, and incubated on ice for 45 min with 50 μl of 5× diluted hybridoma-conditioned medium containing anti-mouse VCAM-1 mAb (M/K 2.7.7) (ATCC). After washing, the cells were incubated for 30 min with 50 μl of 10× diluted FITC-conjugated goat anti-rat IgG (Life Technologies Ltd.). The cells were washed twice, resuspended in 1 ml of medium and analyzed using the Consort 30 program on a FACScan (Becton-Dickinson). The background fluorescence was determined using cells incubated with secondary antibodies only.

*2.5. Nitrite assay*

Nitrite assay was performed as described previously [17]. Cells were cultured in 96-well plates in 100 μl of appropriate culture medium. The medium was replaced with 100 μl of RPMI 1640 supplemented with 2% FCS and antibiotics and containing the factors indicated in each experiment. After 24 h incubation, nitrite concentration in the medium was determined by a microplate assay. 80 μl aliquots of the culture medium were incubated with equal volumes of Griess reagent at room temperature for 10 min. The absorbance at 540 nm was measured with an ELISA reader. Nitrite concentration was determined by using dilutions of sodium nitrite in water as a standard.

*2.6. RNA preparation and Northern blot analysis*

MME were cultured in 94-mm tissue culture plates. After 15 h incubation of the cells with the factors as indicated, total RNA was prepared using phenol extraction method [18]. RNA samples (5 μg) were separated electroforetically in 1% agarose gel under denaturing conditions [19]. RNA was then transferred to Hybond-N membranes (Amersham, UK). The blots were baked at 80°C for 2 h, prehybridized overnight and hybridized at 68°C for 24 h to cDNA probes specific for the murine macrophage iNOS (a gift from Drs. Q.-W. Xie and C. Nathan, New York, NY), murine VCAM-1 (a gift from L. Osborn, Cambridge, MA) and for 18S rRNA (kindly provided by N. Bhownick, Athens, GA). The probes were labeled by the Random Primers DNA Labeling System (Amersham, UK). Non-specifically bound radioactivity was removed by washing the blots in 2× standard saline solution (SSC) at room temperature, followed by two subsequent washes in 2 × SSC/0.1% SDS at 68°C for 30 min each. The blots were then subjected to autoradiography at −70°C using intensifying screens.

*2.7. Nuclear extract preparation and DNA-protein binding assay*

MME cells were cultured in 94-mm culture plates. After the cells reached confluence the medium was changed for RPMI 1640 containing 2% FCS and antibiotics. 24 h later, TNF or ouabain were added to the medium. After 1 h incubation, the monolayers were washed twice with ice-cold PBS, scraped from plates in 0.8 ml of ice-cold PBS and centrifuged at 200 × *g* for 5 min. Nuclear extracts were isolated using the method of Olnes and Kurl [20]. Protein concentration was determined using the Lowry method. Oligonucleotide probe: 5′-AAGTCCGGGTTTTCCCCAACC-3′, containing two repeats of NF-κB binding site, corresponding to -1101 to -1081 bp region of murine c-myc [21] was synthesized by Molecular and Macromolecular Research Center, Polish Academy of Sciences, Łódź, Poland. Aliquots of 0.4 ng (~2 × $10^4$ cpm) of 5′-labeled probe were incubated at room temperature with 5 μg samples of nuclear proteins in the presence of 2 μg of poly(dI-dC) in 10 μl of 10 mM Tris buffer (pH 7.5) containing 50 mM NaCl, 0.1 mM DTT, 1 mM EDTA and 10% glycerol. Some samples were incubated in the presence of 50-fold molar excess of specific cold oligonucleotide probe as a competitor. Protein–DNA complexes were separated in a 4.5% polyacrylamide gel in 0.5× TBE. The gels were dried and exposed to X-ray film at −70°C.

## 3. Results

Exposure of MME to ouabain resulted in the stimulation of VCAM-1 expression on the cell surface as monitored by flow cytometric analysis (Fig. 1). The effect of ouabain was weaker than that of TNF and was similar to the effect of IFN-γ. The expression of VCAM-1 in MME was additionally monitored by the binding of P815 to MME monolayers [16]. The effect of ouabain was dose dependent and the maximal stimulatory effect was observed at a concentration of 0.25 mM (Table 1).

Efficient stimulation of iNOS in MME cells requires a dual signal which can be provided by a combination of TNF and IFN-γ [17]. We found that ouabain may serve as a costimulator in the activation of iNOS expression as measured by the accumulation of nitrite in culture medium (Fig. 2). Ouabain was able to substitute for either of the cytokines in this assay. The simultaneous treatment of MME with TNF and ouabain resulted in a high level of nitrite accumulation, comparable to that obtained when the cells were incubated with TNF and IFN-γ (Fig. 2A). After treatment of MME with ouabain and IFN-γ (Fig. 2B), there was also substantial accumulation of nitrite although the effect was much weaker than the effect of ouabain/TNF. Furthermore, exposure of MME to TNF and

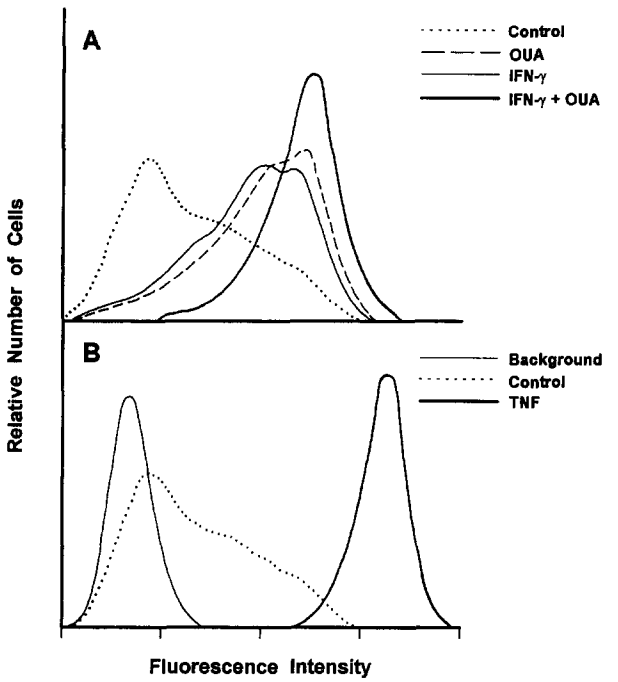


Fig. 1. Flow cytometric analysis of VCAM-1 expression in MME cells. (A) Cells were incubated for 24 h with medium alone (control); ouabain (0.25 mM); IFN-γ (200 U/ml) or with IFN-γ (200 U/ml) in the presence of ouabain (0.25 mM); (B) Cells were incubated for 24 h with medium alone (control) or TNF (2 ng/ml). Background level of fluorescence represents staining of cells incubated only with secondary antibodies (see section 2). Data are from a single experiment representative of 3 experiments.

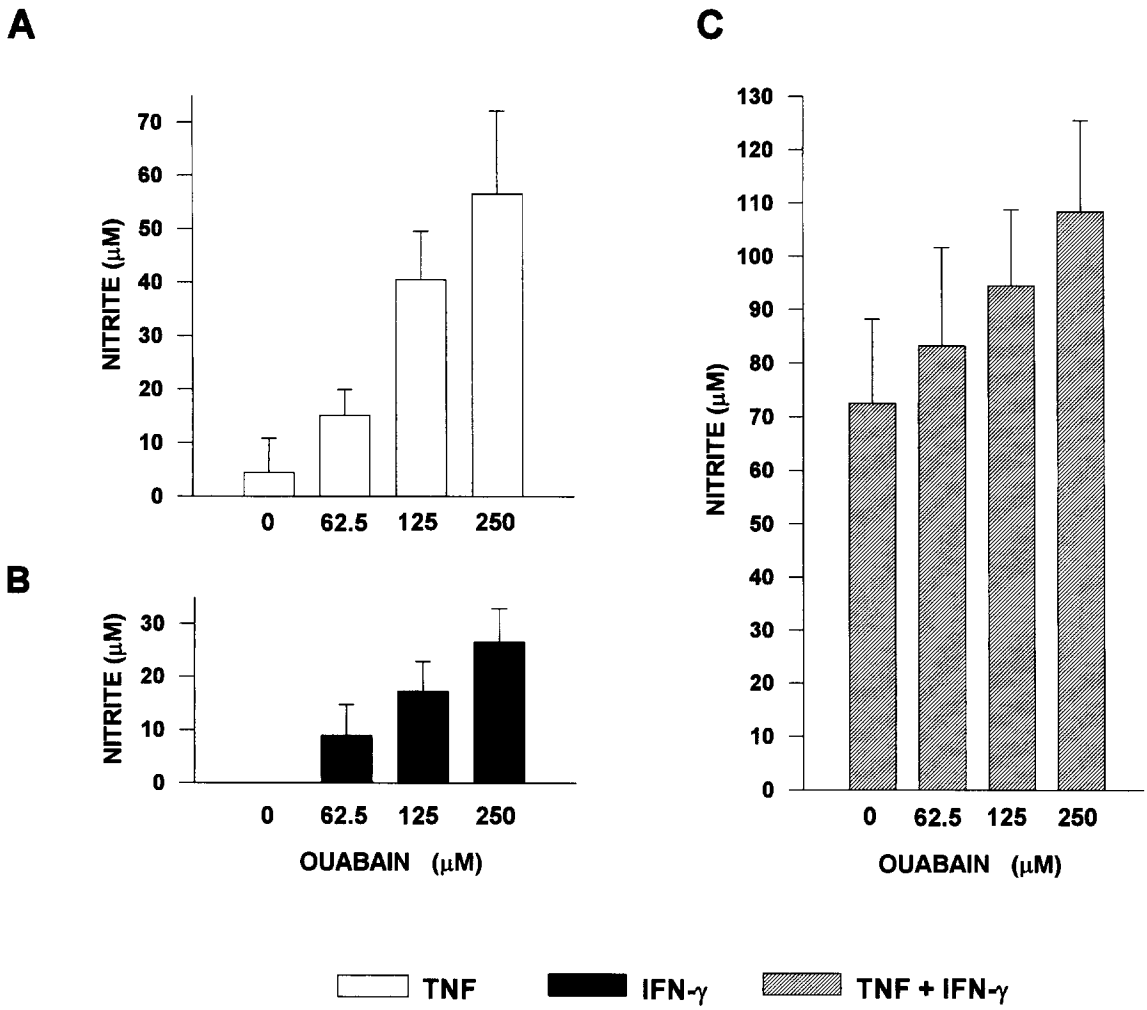


Fig. 2. Accumulation of nitrite in MME culture medium. (A) MME were stimulated for 24 h with TNF (2 ng/ml) in the presence of different concentrations of ouabain; (B) MME were stimulated for 24 h with IFN-$\gamma$ (200 U/ml) in the presence of different concentrations of ouabain; (C) MME were stimulated for 24 h with TNF (2 ng/ml) + IFN-$\gamma$ (200 U/ml) in the presence of different concentrations of ouabain. Bars represent means ± S.D. of 5 experiments and each determination was done in triplicate.

IFN-$\gamma$ in the presence of ouabain led to even higher nitrite production than in cells treated only with cytokines (Fig. 2C). The same range of concentrations of ouabain was effective for stimulation of nitrite accumulation as for enhancement of VCAM-1 expression.

The synthesis of large amounts of nitric oxide by iNOS and significantly increased levels of VCAM-1 in the cell membrane of MME are always preceded by stimulation of the expression of genes encoding these proteins [16,17]. This observation suggested that ouabain is able to stimulate VCAM-1 gene expression and also that this factor could act as a costimulator in the activation of transcription of the iNOS gene. Northern blot analyses of RNA isolated from MME incubated for 15 h with TNF, IFN-$\gamma$ and ouabain confirmed this hypothesis. Ouabain strongly potentiated the effect of TNF on iNOS expression. Moreover, neither ouabain alone nor IFN-$\gamma$ alone was able to induce iNOS expression in MME, but the combination of these two factors resulted in the high level of iNOS mRNA present in these cells (Fig. 3).

The induction of VCAM-1 expression was observed in MME incubated with ouabain alone. The factor also enhanced the stimulatory effect of IFN-$\gamma$ on VCAM-1 expression. The observation that the level of VCAM-1 mRNA in the cells treated with TNF in the presence of ouabain (0.25 mM) is lower than in the cells incubated with TNF alone is not surprising. MME exposed simultaneously to TNF and ouabain produce large amount of NO which is capable of inhibiting VCAM-1 gene expression to some extent [22].

The induction of VCAM-1 and iNOS gene transcription by cytokines involves activation of NF-$\kappa$B [3,4]. We found that ouabain, like TNF, activates NF-$\kappa$B resulting in its translocation to the nuclei of MME cells (Fig. 4).

## 4. Discussion

In the current studies we demonstrated that ouabain activates murine endothelial cells in culture. Ouabain was able to stimulate VCAM-1 expression, potentiate the effect of IFN-$\gamma$ on this process and also provide a complementary signal for either TNF or IFN-$\gamma$ in stimulating iNOS expression. The effect of the drug on iNOS expression was not restricted to endothelial cells since ouabain acted as a costimulator of NO

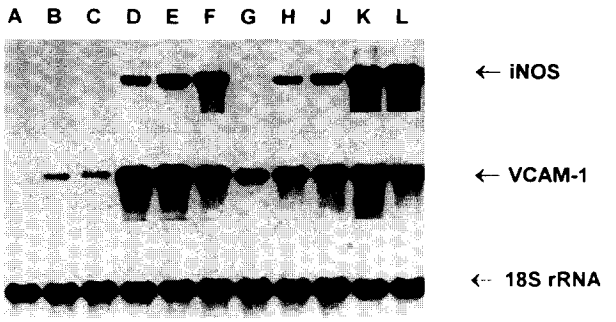


Fig. 3. Northern blot analysis of iNOS and VCAM-1 mRNAs in MME cells. Cells were incubated for 15 hours with medium alone (lane A), with ouabain (0.125 mM) (lane B), ouabain (0.25 mM) (lane C), TNF (2 ng/ml) (lane D), TNF in the presence of ouabain (0.125 mM) (lane E), TNF in the presence of ouabain (0.25 mM) (lane F), IFN-$\gamma$ (200 U/ml) (lane G), IFN-$\gamma$ (200 U/ml) in the presence of ouabain (0.125 mM) (lane H), IFN-$\gamma$ (200 U/ml) in the presence of ouabain (0.25 mM) (lane J), TNF (2 ng/ml) + IFN-$\gamma$ (200 U/ml) (lane K), TNF (2 ng/ml) + IFN-$\gamma$ (200 U/ml) in the presence of ouabain (0.25 mM) (lane L). Equal loading of wells with RNA samples was verified by hybridization with 18S rRNA-specific cDNA probe.

synthesis in rat peritoneal macrophages and P388/D1 murine macrophage-like cell line (data not shown). Activation of NF-$\kappa$B observed in MME exposed to ouabain may be responsible for the stimulatory effects of the drug.

We excluded the possibility that the effects of ouabain originated from its contamination with LPS. We used endotoxin-free preparations of the drug and confirmed that the solutions of ouabain were negative for LPS in a Limulus assay. Moreover, ouabain was able to potentiate the effect of LPS even if LPS was used at very high concentrations (>1 $\mu$M) (data not shown). In addition, another cardiac glycoside, digoxin, had the same effect on VCAM-1 and iNOS expression as ouabain (data not shown).

Although our results indicate that ouabain exerts its effects in MME through the activation of NF-$\kappa$B, the intracellular events that follow the binding of ouabain to $Na^+/K^+$-ATPase are unclear at this time. It is known that elevated $[Na^+]_i$ and a diminished sodium concentration gradient across the plasma membrane may influence other ion exchange systems that could be involved in intracellular signaling. The $Na^+$ gradient is the energy source for the $Na^+$-$Ca^{2+}$ antiport, and thus ouabain might indirectly elevate $[Ca^{2+}]_i$. This mechanism which could explain increased contractility of the heart in response to cardiac glycosides [23], has also been postulated for the effects of these agents in other cells [24,25]. However, it has been shown that in some cell types, biological effects of ouabain are not accompanied by increased $[Ca^{2+}]_i$ [26]. Sage et al. showed that ouabain did not cause elevation in $[Ca^{2+}]_i$ in bovine pulmonary artery endothelial cells [27]. It is unlikely that the stimulatory effect of ouabain in MME cells would be the result of increased $[Ca^{2+}]_i$, since we have previously shown that the elevation in $[Ca^{2+}]_i$ in these cells by various calcium-mobilizing agents had the opposite effect and led to inhibition of cytokine-stimulated VCAM-1 and iNOS expression [17]. Moreover, our preliminary experiments did not show any increase in $^{45}Ca^{2+}$ uptake by MME cells exposed either to cytokines (TNF, IFN-$\gamma$) or to ouabain (data not shown). Furthermore, there appear to be no reports showing that calcium ions activate NF-$\kappa$B. Increased $[Na^+]$ may also influence other ion-exchange systems as for example the $Na^+$-$H^+$antiport which is responsible for maintaining $pH_i$ and which has been shown to take part in regulation of processes involved in growth, differentiation and cell metabolism [28]. We are currently evaluating whether ouabain exerts its effect on MME through an indirect modulation of activity of an ion-exchange systems.

Another possibility which could explain ouabain effects is based on the fact that membrane-coupled ion translocation is an important ATP-requiring process of the cell. Different cells use from 5% to 50% of energy stored in ATP molecules for maintaining the concentration gradients of the ions [29,30]. Inhibition of the $Na^+/K^+$-ATPase would lead to transient disruption in the balance between the rate of ATP synthesis and ATP hydrolysis catalyzed by $Na^+/K^+$-ATPase. This would result in more ATP available to be utilized by protein kinases involved in the activation of transcription factors (including NF-$\kappa$B). The level of phosphorylation of cellular proteins depends on activities of two classes of enzymes: protein kinases and protein phosphatases. It has been shown that inhibition of intracellular phosphatases by okadaic acid leads to increased phosphorylation of numerous proteins involved in signal transduction and mimics the effect of cytokines (or other pro-

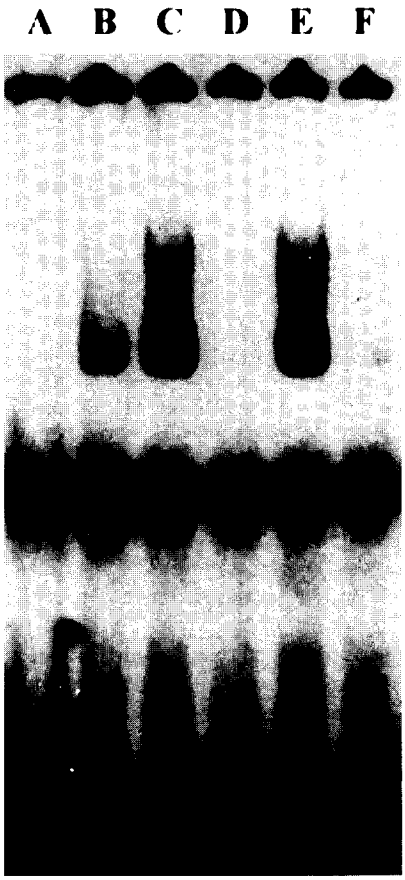


Fig. 4. Gel retardation assay of MME nuclear proteins binding to NF-$\kappa$B. $^{32}$P-labeled oligonucleotide probe was incubated for 30 min with nuclear extracts isolated from MME cells exposed for 1 h to: medium alone (lane B), ouabain (0.25 mM) (lane C and D) or TNF (2 ng/ml) (lane E and F). Nuclear extracts were incubated with the probe in the absence (lane C and E) or presence of 50-fold molar excess of specific cold oligonucleotide probe as a competitor (lane D and F). Lane A contains free probe. Data are from a single experiment representative of 4 distinct nuclear extracts preparations.

Table 1
Adherence of P815 cells to MME monolayers[a]

| Stimulator | % of P815 binding |
|---|---|
| None | 18.0 ± 3.5[b] |
| Ouabain: | |
| 0.062 mM | 25.7 ± 4.9 |
| 0.125 mM | 30.4 ± 3.8 |
| 0.250 mM | 35.9 ± 4.4 |
| TNF 2 ng/ml | 58.6 ± 6.2 |
| IFN-γ 200 U/ml | 29.5 ± 4.2 |

[a] MME monolayers were incubated for 24 h with indicated factors prior to the adhesion assay.
[b] Data represent means ± S.D. of 6 experiments and each determination was done in triplicate.

tein kinases activators) [31]. Similarly, increased intracellular levels of ATP (a substrate for kinases) may lead to the changes in the equilibrium between phosphorylation and dephosphorylation processes and thus promote the activation of transcription factors.

In summary, we have shown that cardiac glycosides are able to stimulate gene expression in endothelial cells through the activation of NF-κB.

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