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Biochimica et Biophysica Acta 1524 (2000) 57–65

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

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Effects of quinacrine on endothelial cell morphology and transcription factor–DNA interactions

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Received 5 September 2000; accepted 7 September 2000

Abstract

Quinacrine has been used for decades and the beneficial effects of this drug are as numerous as its toxic effects. Since endothelial cells (EC) are in many cases the first cells coming in contact with drugs, the effect of quinacrine on certain aspects of EC biology were studied. The presented data demonstrate that quinacrine can have a marked impact on the integrity on EC monolayer without grossly interfering with cell viability. The described impact of quinacrine on EC might explain, at least in part, the toxic effects of this drug observed in the past. Furthermore, quinacrine profoundly effects gene regulation in EC. Quinacrine binds to DNA in a sequence-specific manner. While NF-κB–DNA interactions are not effected, AP-1–DNA binding is blocked by quinacrine. Such differential effects are presumably due to intercalation of quinacrine into the AP-1 consensus element. Preincubation of oligonucleotides resembling this sequence blocked the subsequent binding of nuclear extract containing AP-1 protein(s). Taken together, these data suggest that quinacrine interferes with EC physiology and alters the repertoire of EC to respond to stimuli. Furthermore, the differential effects of quinacrine might be exploited to study and gain additional insight in the involvement of AP-1 and NF-κB in gene regulation.

Keywords: Endothelial cell; Quinacrine; Nuclear factor κB; Activation protein-1

1. Introduction

Quinacrine has been given as a drug for decades for a variety of reasons. One of the earliest, and still the most prevailing common use of quinacrine, is as an antimalarial drug. However, widespread resistance of Plasmodia to this drug has limited its use considerably. Quinacrine and other antimalarials can be beneficial for cutaneous lupus erythematosus and for a number of auto-immune diseases such as rheumatoid arthritis [1,2]. Although the mode of action of quinacrine as an antimalarial is still not completely understood, the drug has been found to influence cells at many levels. Quinacrine interferes with the incorporation of ATP into RNA and DNA as well as with the uptake of adenosine into cells [3]. Many other properties have been assigned to quinacrine, e.g., some tumor cells selectively uptake quinacrine [4], quinacrine can act as a radiosensitizer [5], it effects brain biology [6] and can lead to nucleolar fragmentation [7]. Finally, quinacrine has gained importance lately as an alternative regimen of birth control in countries without access to more expensive alternatives [8].

It has also been demonstrated that quinacrine has many detrimental effects. The drug appears to increase local levels of interleukin-1β, therefore to induce inflammation in malignant pleurisy [9]. Quinacrine may be toxic when administrated intravenously or intraperitoneally in monkeys [10], and has enhancing effects on development of hepatopancreatic lesions [11].

It is also well documented that quinacrine might exert certain anti-inflammatory properties by acting as a phospholipase A_2 inhibitor [12,13] as well as an acetylcholine receptor antagonist [14]. Quinacrine has been shown to reduce infarct size after transient cerebral artery occlusion [15], to attenuate cyclosporine-induced nephrotoxicity in rats [16], to inhibit endotoxin-induced tissue factor expression in endothelial cells [17], to influence E-selectin upregulation [18] and to suppress oxygen radical release from human macrophages [19]. Quinacrine partially precludes complement-dependent polymorphonuclear leukocyte aggregation [20], decreases pulmonary capillary leaks in rats

Abbreviations: EC, endothelial cells; NF-κB, nuclear Factor κB; AP-1, activation protein-1; EMSA, electrophoretic mobility shift assay

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0304-4165/00/$ – see front matter
PII: S0304-4165(00)00140-9

given interleukin-1 [21], inhibits thrombin-induced platelet responses [22], and inhibits the primary proliferative response of cytotoxic T cells to antigens [23].

It is these potentially beneficial effects of quinacrine which lead us to investigate further the effect of quinacrine on the prevention of endothelial cell (EC) activation. EC, lining the inner surface of blood vessels, play a critical role in hemostasis, e.g., by preventing unwanted activation of the coagulation and complement cascades. EC are also critical in the propagation of inflammatory responses by controlling the trafficking of immunocompetent cells [24]. The integrity of the EC layer for example is essential and, while quiescent EC contribute to normal tissue function by, e.g., expressing thrombomodulin and CD39 [25], activated EC contribute to inflammation, e.g., through the expression of adhesion molecules such as P-selectin, E-selectin, VCAM-1, ICAM-1, by the release of cytokines such as interleukin (IL)-1, IL-8, and the upregulation of tissue factor [26]. This study examined the time- and dose-dependent effects of quinacrine on EC morphology and EC activation. Furthermore, the differential effect of quinacrine on two essential transcription factors, nuclear factor κB (NF-κB) and the activation protein-1 (AP-1), were investigated.

2. Material and methods

2.1. Cell culture

Porcine aortic endothelial cells (EC) were isolated and passaged in our laboratory as previously described [27]. In brief, EC were cultured in Dulbecco's modified Eagles medium (DMEM) with 4.5 g/l glucose and supplemented with 10% heat-inactivated fetal bovine serum (Hyclone), L-glutamine and 50 U/ml penicillin/streptomycin. Pig EC from passage 3–7 were used in these experiments. In all experiments cells were used 1–2 days after forming a confluent monolayer.

2.2. Reagents

Quinacrine (6-Chloro-9-[4-diethylamino)-1-methylbutyl]amino-2-methoxy-acridine dihydrochloride, lipopolysaccharide (*Escherichia coli* serotype 055:B5), and MTT (3,4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was from Sigma (St. Louis, MO). Tumor necrosis factor-α (TNF) was from R & D Systems, Minnesota.

2.3. Measuring apoptosis and cell viability

Viability of endothelial cells exposed to quinacrine was confirmed using an MTT assay as described [28]. Apoptosis was analyzed using published methods [29] on a FACScan bench-top model (Becton Dickinson Immunocytochemistry Systems, San Jose, CA) using Cellquest acquisition and analysis software. In short, attached EC were trypsinized and pooled with detached cells, rinsed twice in medium containing serum and suspended in ice-cold PBS. Cells were fixed in 70% ethanol for 3 h and incubated in a solution containing propidium iodide (PI) and RNase A for 1 h.

2.4. Enzyme-linked immunosorbent assay (ELISA) for detection of E-selectin

Cells were seeded in 96-well tissue culture plates coated with gelatin. Confluent cells were treated as described in Section 3. Following this procedure, EC were fixed in ice-cold 0.03% glutaraldehyde at 4°C for 10 min. After three washes, cells were incubated for 1 h with an antibody directed against E-selectin BBA1 (R & D Systems) diluted 1:10 000 in PBS–Tween [27]. As a detection system, a horseradish peroxidase-coupled goat anti-mouse antibody (Pierce) was used and the OD read at 490 nm. All ELISA experiments were performed as triplicates. The integrity of the EC monolayer was confirmed by phase microscopy after each experiment.

2.5. Electrophoretic mobility shift assay (EMSA)

Nuclear extracts from porcine EC were prepared as described [30]. The double-stranded, blunt-ended oligonucleotides used in all experiments were end-labeled using T4 polynucleotide kinase and γ-[^{32}P]ATP. Oligonucleotides for NF-κB (5′-AGT TGA GGG GAC TTT CCC AGG C-3′), AP-1 (5′-CGC TTG ATG ACT CAG CCG GAA-3′) and AP-2 (5′-GAT CGA ACT GAC CGC CCG CGG CCC GT-3′) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). After labeling, 5 μg of nuclear extract were incubated with 100 000 cpm of labeled probe in the presence of 3 μg poly(dI-dC) at 4°C for 30 min followed by separation of this mixture on a 6% polyacrylamide gel in Tris/Glycine/EDTA buffer at pH 8.5. For specific competition, 7 pmol of unlabeled NF-κB/AP-1 oligonucleotides were included; and for non-specific competition, 7 pmol of the double-stranded mutant κB oligonucleotides 5′-AGC TTA GAT TTT ACT TTC CGG AGA GGA-3′ and 7 pmol of the consensus AP-2 element were used. For supershift assays, 1.5 μl of the monoclonal anti-NF-κB p56 subunit antibody (Santa Cruz Biotechnology) was added to the nuclear extract simultaneously with the labeled probe.

3. Results

3.1. Quinacrine induces morphological changes in EC but not necrosis or apoptosis

Treatment of PAEC for extended periods of time with 10 μM of quinacrine leads to gap formation in the cell

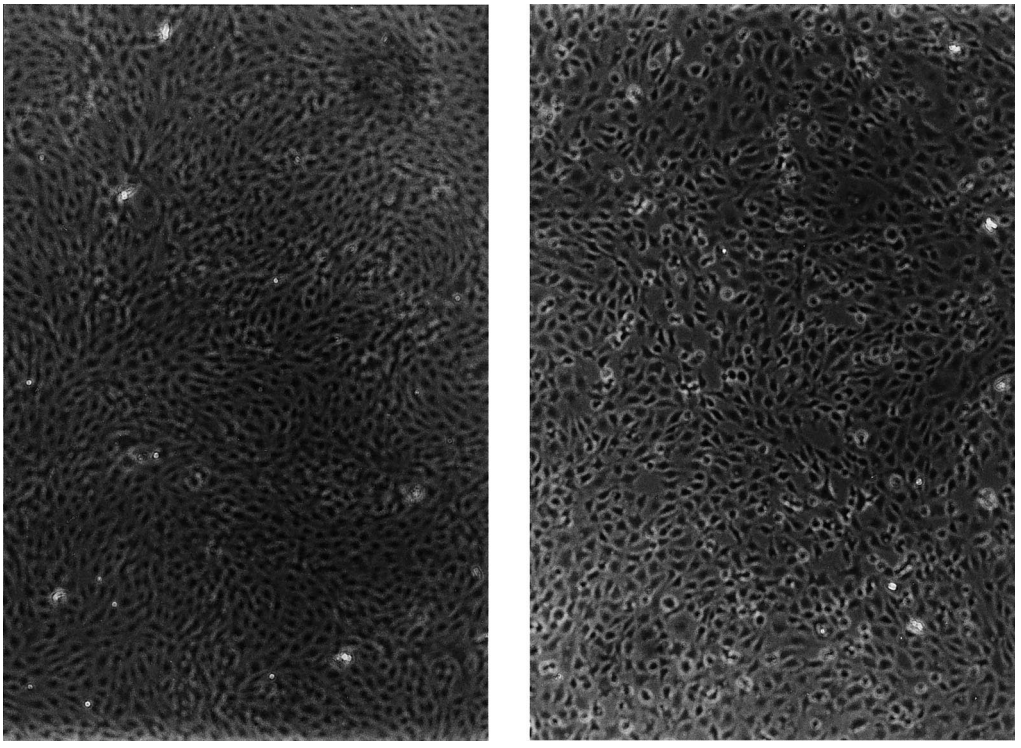

Fig. 1. Quinacrine alters EC morphology. Shown is a comparison of untreated and quinacrine treated EC. The left panel shows control cells, demonstrating an intact monolayer. The right panel shows EC treated with quinacrine (10 μM) for 16 h. Gap formation, retraction of EC and early stages of detachment are clearly visible in cells treated with quinacrine.

monolayer followed by detachment of EC. EC were incubated for up to 72 h with increasing doses of quinacrine (1–30 μM) in complete medium. Phase-contrast microscopy revealed that EC exposed to levels of quinacrine ≥10 μM for 16 h start to retract from each other forming gaps between cells. Fig. 1 shows a comparison of untreated cells and cells treated with quinacrine (15 μM) for 16 h. While untreated cells form a classical monolayer, cells exposed to quinacrine loose their monolayer integrity: they form intercellular gaps, round up, contract, and finally after about 48 h ≥90% of EC were detached from the substrate.

Quinacrine, or the quinacrine induced detachment of EC is not associated with cell death or apoptosis. Viability of EC exposed to quinacrine for 4, 24 and 48 h was tested by the (3-4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) conversion assay. These experiments confirmed that, at the concentrations used in our experiments, quinacrine was not grossly toxic to EC. The results of one representative experiment, where EC were incubated with 2 or 10 μM quinacrine for 24 h are shown in Fig. 2A. While ethanol fixed cells (CONT) cannot convert MTT to formazan, quinacrine-treated cells (QUIN 10, and QUIN 2) are able to convert MTT to a similar degree as untreated cells (MED), confirming the functional integrity of this cells. DNA fragmentation was monitored as a further attempt to assess possible detrimental effects of quinacrine to EC. EC were exposed to quinacrine (10 μM) for 48 h. Following this incubation period, the EC in suspension as well as the remaining adherent cells were harvested and stained with propidium iodide (PI) in order to label DNA and possible DNA fragments. Fig. 2B shows results of one such experiment. EC were left untreated in panel A, in panel B cells were exposed to quinacrine (10 μM) and in panel C, EC were treated with Lipopolyssacharide (LPS) 0.5 μg/ml for 24 h. While EC treated with LPS underwent apoptosis (22 ± 6%), cells incubated with quinacrine for 48 h did not show significant higher numbers of cells undergoing apoptosis (5.1 ± 3%) compared to untreated cells (3.5 ± 5%).

3.2. *Quinacrine differentially affects LPS- or TNF-induced E-selectin upregulation*

Quinacrine has been reported to have certain beneficial effects by preventing EC activation when these cells were exposed to this substance for extended periods of time (≥ 24 h). The effect of 1 and 10 μM quinacrine on TNF- and LPS-induced upregulation of E-selectin was measured. EC were incubated with the indicated amounts of quinacrine for 1 h. Subsequently, quinacrine not taken up by cells was carefully removed by washing the cells with prewarmed medium three times. Removal of quinacrine from the culture medium (after a 1-h exposure) ensured the integrity of the EC monolayer. Cells treated for 1 h only did not show any signs of gap formation. Following a 14 or 22 h incubation period in medium, EC were stimulated with either TNF (5 ng/ml) or LPS (100 ng/ml) for an additional 4 h to allow for the upregulation of E-selectin. Be-

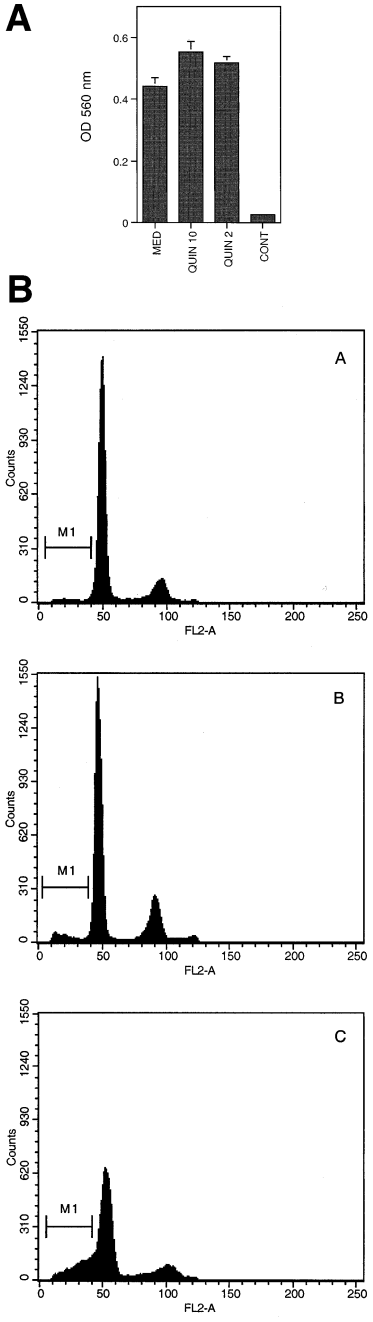

Fig. 2. Quinacrine does not significantly affect viability of EC. (A) A representative experiment where cells were treated with quinacrine (2 and 10 μM) for 24 h before MTT (0.5 mg/ml) was added for 2 additional hours. While ethanol fixed cells (CONT) cannot convert MTT, no decrease in MTT conversion could be observed in cells exposed to quinacrine (QUIN 10, QUIN 2) when compared to cells left untreated (MED). (B) Results of an experiment where EC were exposed to quinacrine (10 μM) for 48 h. FACS analysis was performed to quantitate cells undergoing apoptosis. EC were left untreated (A), treated with quinacrine (B) or, to induce apoptosis, cells were exposed to LPS (0.5 μg/ml) for 24 h (C). In this particular experiment, DNA fragmentation could be detected in 3.9% (A), 5.9% (B) and 21.2% (C) of cells. The bar labeled 'M1' indicates the area of cells considered to undergo apoptosis. ←

fore measuring the levels of E-selectin by ELISA as described in Section 2, cells were carefully washed with PBS and fixed in glutaraldehyde. The results of one representative experiment are shown in Fig. 3. The effects of quinacrine on TNF induced E-selectin upregulation are shown in the upper panel. Exposure of EC to quinacrine suppressed TNF-induced E-selectin upregulation when cells were stimulated with TNF 14 h (Fig. 1A) and 22 h (Fig. 1B) after quinacrine treatment. When EC were treated for 1 h with quinacrine and stimulated 14 or 22 h later with LPS instead of TNF, higher levels of E-selectin were detected on EC. The lower panel in Fig. 3 shows the result of one such experiment. One-hour exposure of cells to quinacrine (1 or 10 μM) enhanced subsequent LPS induced E-selectin upregulation in a dose-dependent manner. When E-selectin was induced by LPS for 4 h at 14 h (Fig. 3C) or 22 h (Fig. 3D) following removal of quinacrine, increased levels could be detected. Similarly, when E-selectin was induced in EC, 64 h after a 1-h exposure to quinacrine (10 μM), the effect of LPS (100 ng/ml) was diminished, while the effect of TNF (5 ng/ml) remained enhanced (data not shown).

3.3. *Quinacrine differentially affects the transcription factors NF-κB and AP-1*

Transcription factors are essential for the regulation of genes, the following experiments describe the effect of

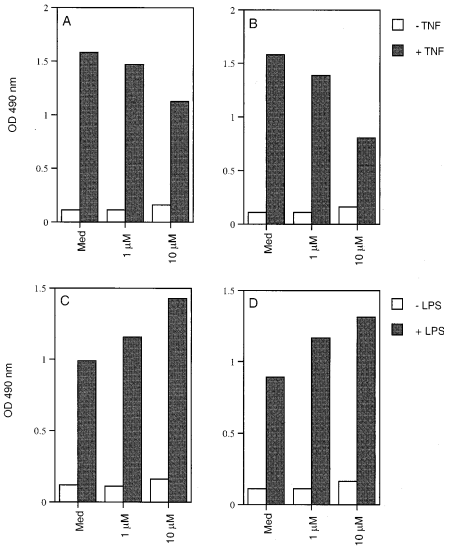

Fig. 3. ELISA experiment demonstrating that pretreatment with quinacrine attenuates TNF-induced E-selectin upregulation but augments LPS-induced surface expression of this molecule. After exposure to quinacrine (10 μM) for 1 h, followed by three wash steps and a period of 14 h (A) or 22 h (B) incubation in medium without quinacrine, EC were treated with TNF (5 ng/ml) for 4 h. Afterwards, cells were fixed with glutaraldehyde and ELISA was performed as described in Section 2. Similar experiments, using LPS as the stimulus to upregulate E-selectin, were also performed. Again, cells were treated with quinacrine (10 μM) for 1 h, washed, and after 14 h (C) or 22 h (D) in medium without quinacrine, stimulated with LPS (100 ng/ml) for 4 h.

quinacrine on two important transcription factors, NF-κB and AP-1. Following a 1-h exposure of EC to quinacrine (10 μM), cells were washed and stimulated with TNF for times ranging from 30 min to 24 h. At the end of indicated incubation times, EC were washed with ice-cold PBS and nuclear protein was extracted as described in Section 2. Nuclear extracts were tested for the presence of NF-κB by electrophoretic mobility shift assay (EMSA). Since in resting cells NF-κB is retained in the cytoplasm, translocation of NF-κB into the nucleus is an indicator of cell activation and is the prerequisite for the subsequent binding to DNA. Similar to NF-κB, AP-1 is an important transcription factor involved in the activation of many genes. Fig. 4A shows the results of an experiment where EC were stimulated for 1 h with quinacrine (10 μM), after changing the medium, these cells were exposed to TNF (5 ng/ml) for 30 min, 5 h, or 24 h. These experiments demonstrated that quinacrine alone did not activate NF-κB to a significant degree. Compared to the extent of NF-κB activation in cells treated with TNF only, there was a consistent but weak increase of NF-κB binding to the consensus NF-κB-element in cells exposed to quinacrine and TNF as opposed to cells treated with TNF alone. The increase in NF-κB activation noted in controls (MED) at later time points (5 and 24 h) compared to cells harvested at 30 min is likely due to basal activation induced by medium changes, etc.

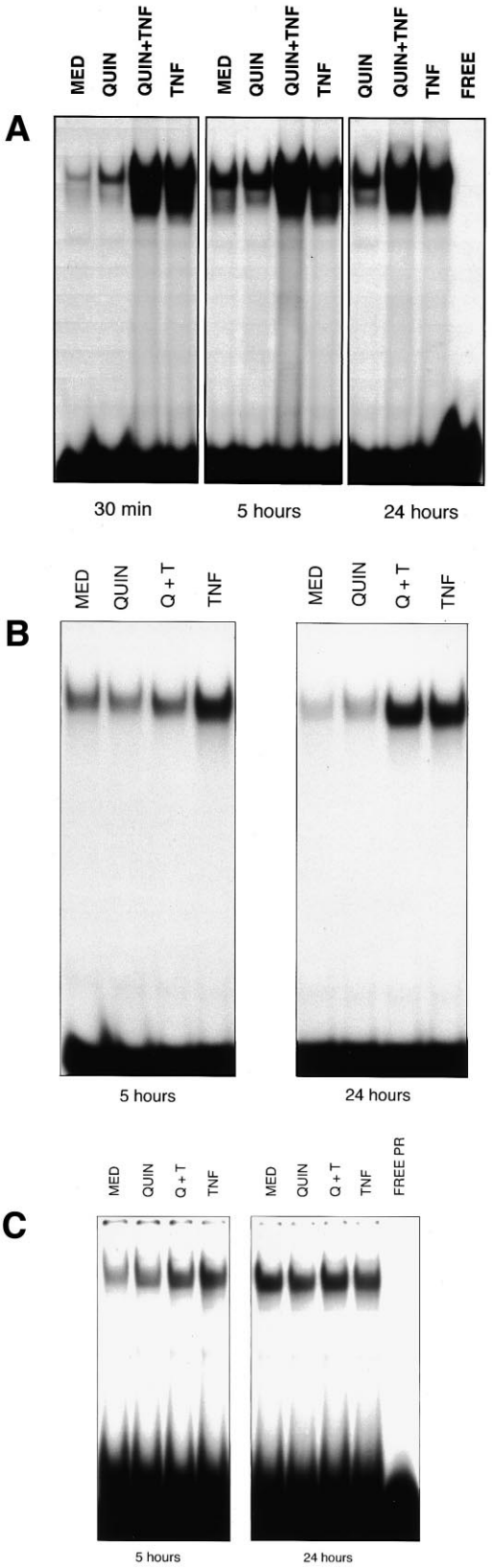

Fig. 4B demonstrates the effect of quinacrine preincubation on TNF-induced AP-1 binding in EC. The constitutive levels of NF-κB inside the nucleus of resting EC are very low, while AP-1 levels are considerable higher. Nevertheless, TNF stimulation of EC magnifies the levels of AP-1 detectable in nuclear extracts. Consistently, at early time points (1–12 h), preincubation of EC with quinacrine prevented activation/binding of AP-1 to the consensus AP-1 element, while at later time points (20–48 h) the effect of quinacrine on AP-1 binding was diminished or entirely absent. Shown in Fig. 4B is one representative experiment where, 5 h after exposure to quinacrine, the effect of TNF (5 ng/ml) on AP-1 activation was prevented. When measured 24 h after exposure to quinacrine, this inhibitory effect was absent. When EC were treated with quinacrine for 1 h, washed and kept in medium without quinacrine, it was noted that, quinacrine diffused out of cells with time, indicating the reversibility of quinacrine binding to DNA (data not shown).

Fig. 4C shows that neither TNF (5 ng/ml), quinacrine (10 μM), nor preincubation of EC with quinacrine fol-

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Fig. 4. Quinacrine inhibits AP-1–DNA interactions but does not influence NF-κB–DNA binding. After a 1-h exposure to quinacrine (10 μM), followed by three washing steps with medium, EC were stimulated with TNF (5 ng/ml) for 0.5, 5, and 24 h. Nuclei were harvested and binding of nuclear protein was analyzed by EMSA. As shown in A, quinacrine did not prevent NF-κB–DNA binding. (B) Binding of protein(s) to a consensus AP-1 element was inhibited by exposure of EC to quinacrine. However, 24 h after quinacrine removal, binding was restored. No noticeable effect of quinacrine (10 μM) or TNF (5 ng/ml) can be seen in control experiments where a consensus CRE element was used instead of NF-κB or AP-1 (C).

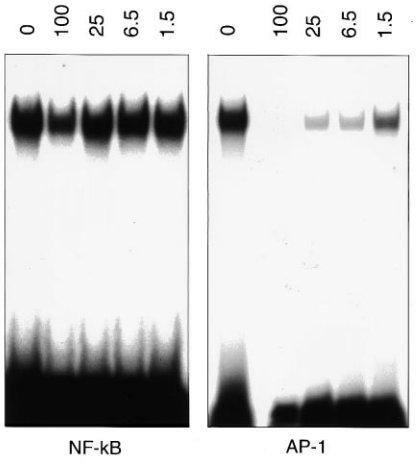

Fig. 5. Quinacrine intercalates DNA in a sequence specific manner and prevents protein binding. Oligonucleotides resembling NF-κB or AP-1 binding sites were incubated with quinacrine, ranging from 1.5 to 100 μM. After 20 min incubation, nuclear extracts, isolated from TNF-stimulated (5 ng/ml for 5 h) EC, was added and the tubes incubated for further 20 min, after which the mixture was separated on a 6% polyacrylamide gel. The left panel shows the results of an EMSA where NF-κB-element oligonucleotides were preincubated with quinacrine. While NF-κB binding overall is unaffected by quinacrine, such pretreatment has a profound effect on AP-1–DNA interactions. As shown in the left panel, 1.5 μM quinacrine significantly affects such interactions while higher doses lead to even greater inhibition.

lowed by TNF (5 ng/ml) for 5 or 24 h had any significant effect on the binding of protein(s) to the consensus cAMP-responsive element (CRE). In lanes labeled 'FREE' in Fig. 4A and 'FREE PR' in Fig. 4C, all components with the exception of nuclear extract were combined and loaded onto the gel. This indicates that the bands in EMSA originate from proteins in the nuclear extract. Further control experiments such as supershift-experiments, competition with unlabeled oligonucleotides, etc., confirmed the specificity of EMSA (data not shown).

3.4. *Quinacrine intercalates DNA; does this influence binding of transcription factors?*

Although quinacrine is known to bind to DNA, the nature of this interaction, the so-called intercalation of quinacrine between the base pairs of DNA, is not well defined. A series of experiments were designed to investigate whether direct DNA-quinacrine interaction are responsible for the differential effect seen on protein–DNA interactions (Fig. 4). The same oligonucleotides, representing the consensus sequence for NF-κB and AP-1 which were used in the EMSA experiments, were used for these experiments. These oligonucleotides were incubated with increasing doses of quinacrine (ranging from 1.5 to 100 μM) in bandshift reaction buffer for 20 min. Subsequently, nuclear extract, isolated from EC stimulated for 5 h with TNF (5 ng/ml), was added to the tubes containing the oligonucleotides for NF-κB, AP-1 ± increasing amounts of quinacrine. After an additional incubation period, to allow for protein–DNA binding (20 min at 4°C), these mixtures were separated on a 6% polyacrylamide gel.

As shown in Fig. 5 (left panel), preincubation of the consensus NF-κB-element with quinacrine did not affect NF-κB–DNA binding. Only when very high doses of quinacrine (100 μM) were used could a slight decrease in NF-κB–DNA binding be noted. While NF-κB–DNA and protein–CRE interactions were not significantly effected by quinacrine, AP-1–DNA interactions were dramatically altered by preincubation of the AP-1 consensus binding element with quinacrine. As shown in Fig. 5 (right panel) preincubation of the AP-1 consensus binding element with quinacrine inhibited the subsequent binding of AP-1. While 100 μM of quinacrine leads to complete inhibition of protein–DNA binding, even a very low level of quinacrine (1.5 μM) inhibited protein–DNA binding significantly.

Preferential binding of quinacrine to DNA is well described [31–34]. Nevertheless, the effects of quinacrine demonstrated in Fig. 5 could also be due to a higher affinity of quinacrine to AP-1 proteins, thereby blocking protein–DNA interactions. To further support our hypothesis that it is the quinacrine–DNA intercalation which blocks subsequent protein–DNA binding, we set out to test whether quinacrine binds with higher affinity to the AP-1 sequence. We utilized the fluorescence properties of quinacrine to measure quinacrine–DNA coupling. These experiments enabled us to examine whether quinacrine binds preferably to oligonucleotides resembling the AP-1 binding sequence. Equal amounts of NF-κB and AP-1 oligonucleotides were incubated with quinacrine (500 μM) for 1 h. Following dialysis (48 h), quinacrine fluorescence associated with DNA was measured (excitation was set at 450 nm and emission was measured at 520 nm). The amount of quinacrine was calculated using fluorescence intensities of known quantities of quinacrine. While in vials containing the NF-κB oligonucleotides, 631 ± 38.4 nM ($n = 4$) of quinacrine was found, the fluorescence of quinacrine bound to AP-1 was calculated to be equal to 818 ± 92.6 nM ($n = 4$) of quinacrine.

4. Discussion

EC lining blood vessel walls are critically important for proper function of all vascularized organisms. Not only does EC serve a barrier function, but these type of cells are, among other functions, actively involved in controlling the movement of circulating micro- and macromolecules, as well as the migration of cells out of from and into the surrounding tissue. Therefore, EC play a key role in the progression, control, and resolution of many pathological conditions. In most cases EC are among the first cells to come in contact with drugs whether they are administrated intravenously or though other ways. As a result, EC are a target of many untoward as well as desired

effects of drugs. Although quinacrine has been used in humans for more than 40 years there have been very few studies investigating the effect of this drug on EC.

It was our goal to better understand events involved in EC activation with the objective of finding novel means of controlling unwanted EC activation. Quinacrine has been reported to have many beneficial effects. Prolonged ($\geq$ 24 h) exposure to quinacrine has been described to prevent complement-induced lysis of EC. This effect was thought to be linked to quinacrine induced upregulation of 'protective' genes (F.H. Bach, personal communication). Other reports demonstrated that quinacrine inhibits the upregulation of certain adhesion molecules on EC and suppresses activation of tissue factor upregulation in monocytes and EC [17,35]. Although many of the effects were thought to be due to an inhibitory effect of quinacrine on phospholipase A_2 [13], it has become evident that quinacrine affects cells at many levels. Furthermore, is has become apparent that quinacrine does not act primarily as an inhibitor of phospholipase A_2, [14,36]. Such reports lead to the experiments described in this manuscript.

The results of this study demonstrated that treatment of EC with quinacrine for extended periods of time can significantly affect the integrity of EC monolayers (Fig. 1). These findings are important since such effects might explain to the reported toxic effects of this drug, e.g., a single intravenous injection was lethal to monkeys [10], and toxic to rodents and dogs [37,38]. Interruption of EC integrity exposes tissue factor on underlying smooth muscle cells [39] can lead to edema, hemorrhage, thrombosis, organ failure, shock and death. In the present study (Fig. 2), retraction of EC is not associated with EC death, nor does quinacrine induce apoptotic events in EC. These data are supported by studies of others suggested that quinacrine, is not grossly toxic. However, one report demonstrated that treatment of immortalized NIH 3T3 cells with quinacrine might trigger the induction of apoptosis by blocking cellular signal transduction [40]. That such an effect can be specific for cancer cells is demonstrated in studies showing that AP-1 inhibition restrained tumor cell proliferation, without affecting the growth of noncancerous cells [41,42].

Also in this study, the integrity of the EC layer was preserved when quinacrine was removed from culture medium after a 1-h exposure. Presumably, such a procedure leads to lower intracellular concentration of quinacrine since it is likely that quinacrine diffuses out of cells. Diffusion of quinacrine is also the most likely explanation for the results shown in Fig. 4B where quinacrine preincubation, followed by washing steps, inhibited binding of AP-1 when measured at early time points, but did not prevent AP-1 binding at later time points (24 h after removal of quinacrine). That quinacrine, over time, actually diffused out of cells could easily be confirmed by monitoring the increasing levels of quinacrine in the medium.

We demonstrated differential effects of quinacrine on TNF- vs. LPS-induced E-selectin upregulation. While TNF-induced E-selectin upregulation is partially suppressed, LPS-induced activation of this adhesion molecule is consistently enhanced (Fig. 3). Similar inhibitory effects of quinacrine on TNF-induced E-selectin upregulation have been reported in the past, but effects of quinacrine on LPS-induced expression of adhesion molecules have not yet been demonstrated. Whereas previously it had been thought that inhibition of PLA_2 might mediate the inhibitory effects, it became clear that arachidonic acid metabolites were not involved in early events leading to E-selectin upregulation. Without further investigations, clearly outside the scope of this project, we cannot offer a single or simple explanation for this phenomenon since quinacrine might interfere with gene regulation at several levels. Nevertheless, the observed differential effects might be exploited further to investigate differences between TNF- and LPS-induced pathways leading to E-selectin upregulation.

To better understand the mode of action of quinacrine, extensive studies have already been carried out. Such experiments demonstrated that quinacrine intercalates DNA. Many of these studies have been performed using native DNA, polynucleotides, or dinucleotides complexes [31,43]. This work demonstrated sequence-specific affinities of quinacrine to DNA without investigating physiological effects of specific binding. Since the specificity of such interactions were expected to alter preferentially the expression pattern of some genes, it was appealing for us to investigate the effect of quinacrine on the binding of transcription factors to their cognate promoter sequences. AP-1, and even more so NF-κB, are two of several transcription factors involved in the regulation of many genes [44–46]. NF-κB in particular has been shown to be involved in EC activation as translocation of NF-κB and subsequent NF-κB–DNA binding has been shown to be required also for the activation of many genes [47]. But there is little doubt that transcription factors are involved in 'housekeeping' functions and are necessary to maintain cell integrity in general, e.g., maintenance of adhesion molecules thereby ensuring contact to substrate as well as to neighboring cells. Fig. 4A and Fig. 5 demonstrate that quinacrine does not interfere with NF-κB–DNA binding. This also excludes the possibility that quinacrine inhibits, as demonstrated (Fig. 3), the TNF-induced E-selectin upregulation by blocking NF-κB activation. This is important since many drugs have been shown to act by suppressing the activation of this important transcription factor in one way or another. Although it is also possible that, compared to TNF-induced EC activation, LPS stimulation leads to the assembly of a different set of proteins in the promoter region of the gene encoding E-selectin, and that quinacrine modifies this assembly in different ways resulting in respective enhancement of suppression of E-selectin expression.

One could argue that quinacrine might attach to pro-

Table 1
Presence of high quinacrine binding sequences in oligonucleotides resembling the NF-κB (AGT TGA GGG GAC TTT CCC AGG C) and AP-1 (CGC TTG ATG ACT CAG CCG GAA) consensus sequence (core; underlined) as well as the number of such pairs found in the undivided sequences (long)

	AP-1 (long)	NF-kB (long)	AP-1 (core)	NF-kB (core)
G–C	4	1	0	0
T–A	1	0	1	0
C–A	2	2	2	1
T–G	2	2	2	0

teins and therefore prevent transcription factor–DNA interactions. Such mechanisms seem to be an unlikely explanation for our findings, as there are many reports demonstrating the DNA specificity of quinacrine. Our findings of specific quinacrine–DNA intercalation is also supported by data of others, which demonstrated a sequence-specific binding of quinacrine to DNA [43]. These authors found that among the neighbor base sequences, C–G, T–G, C–A and T–A seem to bind quinacrine stronger than the remaining sequences. In particular the sites where a G–C base pair is involved were found to display higher affinities. A comparison of the DNA–oligonucleotide sequences used in our experiments shows that the AP-1 oligonucleotide contains significantly more of such high quinacrine binding sites than the NF-κB element (Table 1). This becomes even more obvious when one compares the core sequence of these two oligonucleotides. While the NF-κB binding site contains one high quinacrine binding base sequence, there are five in the AP-1 oligonucleotide, among them is the C–A base sequence which, when mutated, prevents the binding of the AP-1 complex altogether. What is clearly demonstrated in this study is that quinacrine binds to the consensus AP-1 sequence, thereby blocking access of proteins to DNA and most likely modifying or preventing the regulation of AP-1-dependent genes. Furthermore, these experiments suggest that such binding may be reversible since, following removal of the drug, the intracellular concentrations of quinacrine diminishes and AP-1 binding can be restored.

These data show that quinacrine specifically blocks the access of AP-1 proteins to DNA, while not interfering with NF-κB–DNA interactions. This makes it likely that many of the previously described effects of quinacrine are due to similar, differential effects on gene regulation. For example, it has been shown that quinacrine enhances cell killing by X-rays due to inhibition of genes involved in the repair of sublethal damage [5]. The toxic effects of quinacrine are as numerous as its beneficial effects, the experiments shown in this study add some useful additional data, but make it also appealing to speculate that the underlying mechanism of the mode of action of quinacrine is the sequence-specific binding to DNA, resulting in the many described dose- and time-dependent effects this drug.

In summary, the presented data show that EC readily take up quinacrine and that this drug has affects on numerous physiological processes in EC. The data demonstrate that physiologically relevant doses of quinacrine can dramatically alter EC monolayer integrity thereby possibly contributing to the previously reported detrimental effects of this drug. One of the underlying mechanisms for the observed changes seems to be the specific and differential binding of quinacrine to DNA. While quinacrine does not prevent NF-κB–DNA interactions, it specifically prevents AP-1–DNA binding. Therefore, quinacrine interferes with EC physiology and alters the repertoire of EC responses to various stimuli. Furthermore, this differential effect of quinacrine might be used to study the involvement of AP-1 and NF-κB in gene regulation.

Acknowledgements

The author thanks Dr F.H. Bach for financial support of parts of this work.

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