

Effects of Nicotine on PTHrP and PTHrP Receptor Expression in Rat Coronary Endothelial Cells

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Key Words

Apoptosis • Cholinergic receptors • bcl-2

Abstract

Aims: The study was aimed to investigate whether nicotine affects endothelial expression of PTHrP and PTHrP receptor, a peptide system involved in endothelial protection against apoptosis. **Methods:** Isolated and cultured rat coronary endothelial cells were used. Immunoblot techniques were used to study activation of mitogen-activated protein (MAP) kinases and to quantify PTHrP and PTHrP receptor expression. Real-time RT-PCR was used to quantify PTHrP, PTHrP-receptor, bcl-2, and bax mRNA expression. The rate of apoptosis was determined by HOE33258 staining and confirmed by quantification of the bcl-2-to-bax ratio. *In vitro* data were compared to hearts from rats exposed to cigarette smoking. **Results:** Nicotine induced PTHrP protein expression at nanomolar levels and small increases of PTHrP release ($\approx 8\%$). Antagonists directed against the $\alpha 7$ subunit of cholinergic receptors, the most prominent isoform, attenuated nicotine-dependent increases of PTHrP expression.

This effect of nicotine was p38 MAPK dependent. Nicotine at micromolar concentrations reduced PTHrP receptor expression. *In vitro* and *in vivo* we found a correlation between PTHrP receptor expression and bcl-2 expression. **Conclusion:** Nicotine induces PTHrP expression in endothelial cells but excessive concentrations of nicotine reduce PTHrP receptor expression thereby attenuating any protective effects of PTHrP against apoptosis.

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Introduction

Nicotine is a natural alkaloid that can be isolated from the leaves of *Tabacum nicotiana*. Nicotine enfold its physiological activity by activation of receptors that belong to the acetylcholine receptor family, namely nicotinic acetylcholine receptors (nAChR). They are built up as pentamers composed of one out of nine different α subunits and one out of three different β subunits. Noteworthy, nAChRs are expressed on non-neural cells, such as endothelial cells. A couple of quite different effects of nicotine on endothelial cells have been described so far. Nicotine stimulates DNA synthesis and proliferation in vascular endothelial cells [1], it enhances the expression

of ICAM-1, VCAM-1, and E-selectin in human endothelial cells [2, 3]. On the other hand there are also conflicting data in the literature. Nicotine inhibits nitric oxide signalling in cultured human endothelial cells and antagonised the effect of bFGF on endothelial cell proliferation [4, 5]. In general, nicotine seems to display long-term effects that are often in opposition to its short term effects [6].

Nicotine is also one out of a few thousand different compounds released by cigarette smoke. Therefore, nicotine-dependent changes in endothelial protein expression may be part of long-term effects of smoking. Smoking is responsible for approximately 140.000 deaths per year with cardiovascular disease being the second placed reason [7, 8]. Recently we studied the effect of smoking on spontaneously hypertensive rats [9]. Surprisingly, we observed an up-regulation of parathyroid hormone-related peptide (PTHrP) in left ventricles. As endothelial cells are the main source of PTHrP in ventricles this most likely represents up-regulation of endothelial-derived PTHrP. Noteworthy, PTHrP can be considered as a cardioprotective protein reducing the susceptibility of endothelial cells against apoptosis and increasing cardiac function [10, 11]. On the other hand nicotine has been shown to induce pulmonary fibroblast trans-differentiation via down-regulation of PTHrP receptors [12]. All these studies prompted us to hypothesize that nicotine affects endothelial PTHrP and/or PTHrP receptor expression. This will explain some of (patho)physiological effects of nicotine. The understanding of cellular mechanisms by which nicotine influences the endothelial PTHrP/PTHrP receptor system is therefore of importance for the understanding of the smoking-dependent pathophysiological changes in the vascular bed. Such studies may also allow a better understanding of the therapeutic potential of nicotine. This study was aimed to investigate the effect of nicotine on endothelial PTHrP expression and to investigate whether PTHrP is part of nicotine effects on endothelial cells.

Material and Methods

Cells and cell culture

Male Wistar rats (250-300 g) were used for the isolation of coronary endothelial cells. They were isolated as described before and grown for 1 or 2 days before use [13]. As reported previously, the purity of these cultures was >95% endothelial cells, as determined by the uptake of acetylated low-density lipoprotein labelled with 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate, contrasted with <5% cells that were positive for α -smooth muscle actin [14].

Immunoblots

Samples of endothelial cells were prepared for immunoblotting as described previously [15]. Samples were loaded onto a 12.5% SDS-polyacrylamide gel electrophoresis and blotted onto membranes. Blots were incubated first with an antibody directed against PTHrP (antibody GF08; Calbiochem, Bad Soden, Germany) or PTHrP receptor 1 (antibody 3D1.1; Upstate biotechnology, Eschborn, Germany) and then with an anti-mouse IgG antibody coupled to alkaline phosphatase. Samples were re-blotted on separate sheets and the band corresponding to actin was used as a loading control (antibody PC612; Calbiochem, Bad Soden, Germany).

Immunoblots were scanned and densitometrically analyzed via ImageQuant (Amersham Bioscience Inc., Piscataway, NJ).

PTHrP release

To determine the release of PTHrP from cultured endothelial cells they were washed twice with phosphate buffered saline and then incubated for 12 h with a protein-free medium (composition as follows (mM): NaCl 120; NaHCO₃ 24, KCl 2.7, NaH₂PO₄ 0.4, MgCl₂ 1, CaCl₂ 1.8, glucose 5; pH 7.4). At the end of the incubation period the supernatant was collected, 100 μ l desoxycholate (5 mM) was added and the samples were placed on ice for 15 min. Thereafter trichloroacetic acid (100 %) was added to all samples for another 30 min and all samples were placed again on ice. Finally, the protein was pelleted by centrifugation, resolved in 35 μ l Laemmli buffer and 10 μ l Tris/HCl (pH 9.5) and heated for 5 min at 95°C. Samples were stored until use in a refrigerated and analyzed as cell samples (see above).

Real-time RT-PCR

The steady state mRNA levels of PTHrP, PTHrP receptor 1, bcl-2 and bax were quantified via real-time RT-PCR using iQ SYBR Green supermix (Bio-Rad, Germany) as described previously in greater detail [16]. Hypoxanthine phosphoribosyl transferase (HPRT) was used as a housekeeping gene to normalize sample contents. Primers used for determination had the following sequences: PTHrP forward: CGG TGT TCC TGC TGA GCT A, reverse: TGC GAT CAG ATG GTG AAG GA; PTH-rP receptor: forward: GGC TGC ACT CGA CGC GCA A, reverse: TTG CGC TTG AAG TCC AAC GC; HPRT forward: CCA GCG TCG TGA TTA GTG AT, reverse: CAA GTC TTT CAG TCC TGT CC; bcl-2 forward: TTC TTT CCC CGG AAG GAT GG, reverse: GTC TGC TGA CCT CAC TTG TG; bax forward: CCT GAG CTG ACC TTG GAG CA, reverse: CCT GGT CTT GGA TCC AGA CA. Quantification was performed as described before [16]. In order to show the mRNA expression of nicotine α -subunits in endothelial cells, RNA preparations of endothelial cells were amplified as above but finally loaded onto 2 % (wt/vol) agarose gels, stained with SYBRsafe and visualized under an UV-illuminator. For these studies the following primers were used according to the previous published data [17]: α 2: forward: CTT CGG TGA AGG AAG ACT GG, reverse: GGA GCC AAC ATG AAG GAC AT; α 3: forward: CGC CTG TTC CAG TAC CTG TT, reverse: CTT CAG CCA CAG GTT GGT TT; α 4 forward: GAC TTT GCA GTC ACC CAC CT; reverse: CGG CTA

TGC ATG CTC ACT AA; $\alpha 5$: ACC CTA CCA ATT TGC AAC CA, reverse: GAC CCA AAG ACC CAT TCT GA; $\alpha 6$ forward: TGG TGT TAA GGA CCC CAA AA, reverse: GCT GCT GGC TTAACC TCT TG; $\alpha 7$ forward: ACC CTC CAT AGG ACC AGG AC, reverse: GGA GGC TGT ACA AGG AGC TG; $\alpha 9$ forward: CGT GGG ATC GAG ACC AGT AT, reverse: TCA TAT CGC AGC ACC ACA TT; $\alpha 10$ forward: TCT GAC CTC ACA ACC CAC AA, reverse: TCC TGT CTC AGC CTC CAT GT.

Apoptosis assay

To quantify apoptosis, the medium was removed and replaced by 1 ml of PBS with the addition of HOE33258 (5 μ g/ml) at 37°C for additional 30 min. Cultures were analyzed in a fluorescence microscope, and apoptotic cells were identified by clear nuclear-chromatin condensation as previously described [10]. Propidium iodide (5 μ g/ml) was used to counterstain the cells for necrotic cell damage. At least 5 images were counted per culture dish and a total of 5 culture dishes were counted. The average number of total cell counted was 2551 \pm 145 cells for each condition.

Statistics

Data are given as means \pm s.e.m. from n different culture preparations. Statistical comparison between groups was performed by two side one-way analysis of variance and use of Student-Newman-Keuls test for post hoc analysis. A p value of 0.05 was considered as statistically significant. Comparisons between two groups were performed by means of a t-test for independent samples with a critical value equal to 0.05.

Results

Effect of nicotine on PTHrP expression and release

To determine the effect of nicotine on the expression of PTHrP in endothelial cells, we first incubated microvascular endothelial cells for 24 h with nicotine (10 nmol/l – 10 μ mol/l). Nicotine increased PTHrP protein expression. Fig. 1A shows a representative immunoblot indicating a strong PTHrP band at approximately 50 kDa in cells incubated with nicotine. Fig. 1B shows a quantitative summary of the concentration response relationship. A bell shaped concentration response curve was obtained with a maximal effect at 100 nmol/l. At this concentration nicotine caused a 5.4-fold increase of PTHrP protein expression. However, PTHrP mRNA expression did not increase (1.13 \pm 0.07-fold vs. control; n=4, n.s.).

Identification of acetylcholine receptor subtypes and signalling

In order to identify the acetylcholine receptor subtype that is responsible for the observed effect of nicotine on

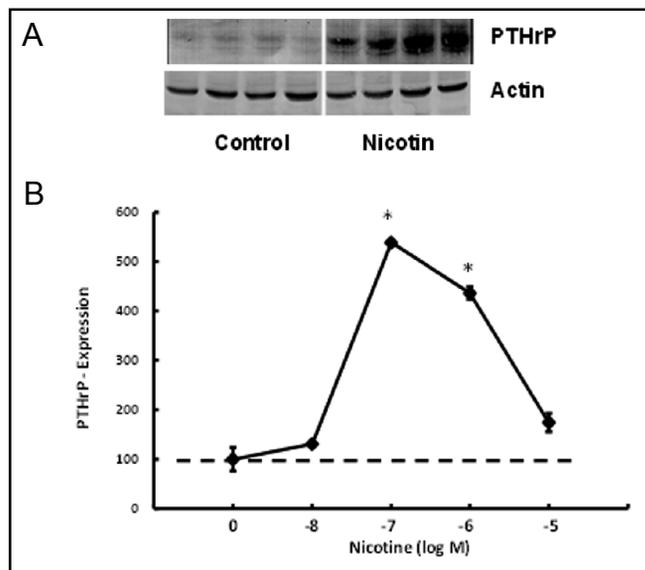


Fig. 1. Induction of PTHrP protein expression in microvascular endothelial cells by nicotine. A) Representative immunoblot from samples of endothelial cells cultured under control conditions or in the presence of 100 nM nicotine for 24 h. B) Concentration-response curve for nicotine-dependent induction of PTHrP protein expression. Data are means \pm S.D. from n=4 cultures. *, p<0.05 vs. control.

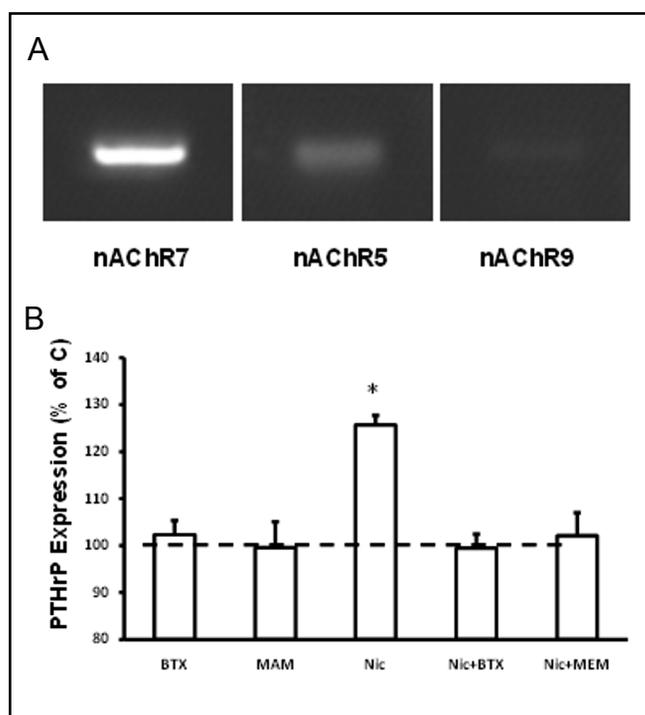


Fig. 2. Contribution of $\alpha 7$ -dependent effects to nicotine-dependent PTHrP induction. A) Representative agarose gels from RNA samples of three different α subunits. B) Induction of PTHrP protein expression by nicotine (30 nM) in the presence and absence of nicotinic receptor antagonists (MEM, mecamylamine, 1 μ M; BTX, bungarotoxine, 1 μ M). Data are means \pm S.D. from n=4 cultures. *, p<0.05 vs. control.

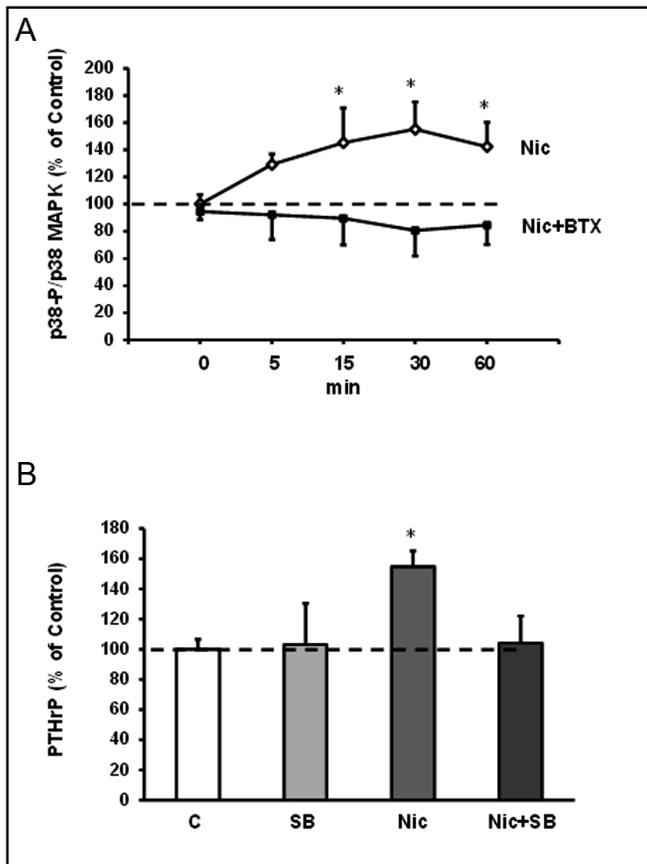


Fig. 3. Contribution of p38 MAP kinase activation to nicotine-dependent PTHrP induction. A) Time-dependent phosphorylation of p38 MAP kinase by nicotine (30 nM) and its inhibition by bungarotoxin (1 μ M). B) Effect of p38 MAP kinase inhibition (SB, SB202190, 10 μ M) on nicotine (30 nM)-dependent induction of PTHrP. Data are means \pm S.D. from n=4 cultures. *,p<0.05 vs. control.

endothelial PTHrP expression we analyzed the expression of the eight possible α subunits of acetylcholine receptors by RT-PCR. As shown on Fig. 2A, a strong band corresponding to the $\alpha 7$ subunit, a weaker expression of the $\alpha 5$ subunit, and small expression of $\alpha 9$ subunits was monitored. No transcripts were found for $\alpha 2$. Amplification of $\alpha 3$, $\alpha 4$ gave non-specific bands, $\alpha 6$, and $\alpha 10$ subunits displayed very weak signals. These data suggest that the $\alpha 7$ subtype is the main isoform expressed in coronary microvascular endothelial cells of rat hearts. Based on these initial studies we investigated the effect of mecamylamin, a non-subtype specific antagonist of nicotinic receptors, and of bungarotoxin, a specific $\alpha 7$ receptor antagonist, on the nicotine-dependent increase of PTHrP expression. As shown in Fig. 2B, both antagonists attenuated the nicotine effect. Collectively these data argue for an $\alpha 7$ -dependent effect of nicotine on endothelial PTHrP expression.

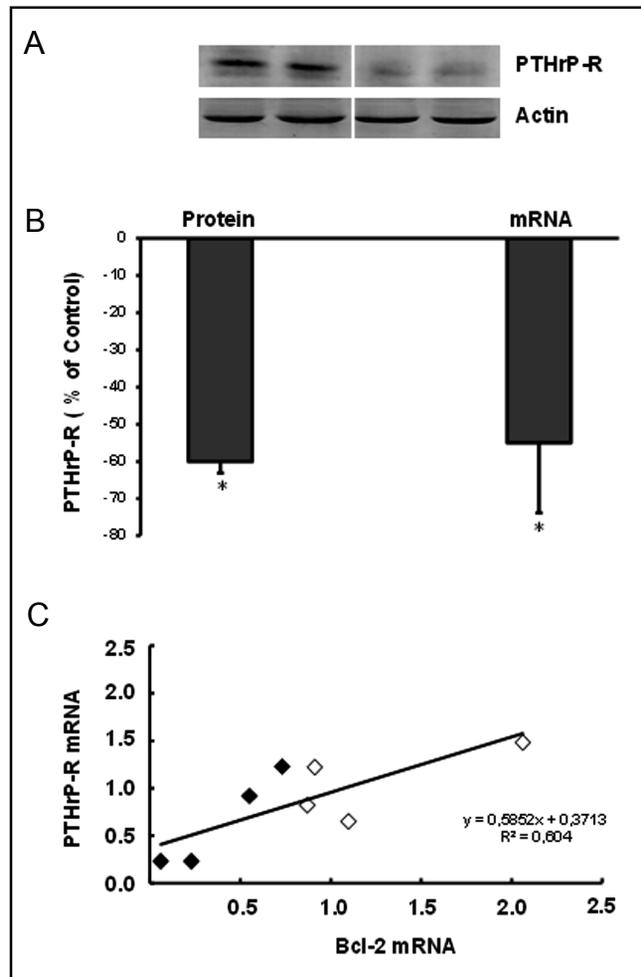


Fig. 4. Effect of nicotine (1 μ M) on PTHrP receptor expression. A) Representative immunoblot indicating down-regulation of PTHrP receptor expression. B) Quantitative analysis of PTHrP receptor protein and mRNA expression. Data are means \pm S.D. from n=4 cultures. *,p<0.05 vs. control. C) Correlation of bcl-2 and PTHrP receptor expression in endothelial cells cultured under control conditions (open symbols) or nicotine (closed symbols).

Next we investigated the participation of signal transduction pathways involved in the nicotine-dependent effect. Among the three major MAP kinase pathways p42/p44 (ERK), c-JNK, and p38 MAP kinase, only the latter one seems to be activated because only here we found a phosphorylation of the kinase in a nicotine-dependent way (Fig. 3A). This effect was again attenuated in co-presence of bungarotoxin (Fig. 3A). Subsequently, it was investigated whether SB202110, a p38 MAP kinase inhibitor, attenuates the effect of nicotine on PTHrP expression. As shown in Fig. 3B that was indeed the case.

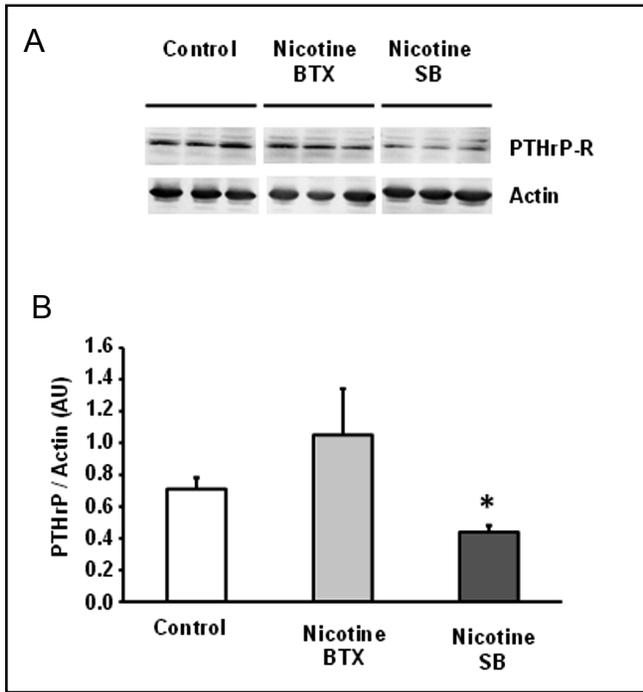


Fig. 5. Effect of bungarotoxin (1 μ M) and SB202190 (10 μ M) on nicotine (1 μ M)-dependent PTHrP receptor down-regulation. A) Representative immunoblot indicating down-regulation of PTHrP receptor expression by nicotine in co-presence of SB202190 (SB) but not in co-presence of bungarotoxin (BTX). B) Quantitative analysis of PTHrP receptor protein expression. Data are means \pm S.D. from n=3 cultures. *,p<0.05 vs. control.

Effect of nicotine on PTHrP receptor expression

Next, we evaluated the effect of nicotine on PTHrP receptor expression. Nicotine (1 μ M) caused a robust reduction of PTHrP receptor protein expression (Fig. 4A) without affecting its expression at lower concentrations. Moreover, the reduction of PTHrP receptor protein expression was accompanied by a corresponding down-regulation of PTHrP receptor mRNA (Fig. 4B). We have recently shown that smoking increases left ventricular expression of PTHrP in spontaneously hypertensive rats [9]. We now re-evaluated these samples and analyzed the expression of PTHrP receptor and bcl-2. The PTHrP receptor mRNA expression was significantly reduced in these samples (0.47 \pm 0.26-fold, p=0.015) as well as that of bcl-2 (0.63 \pm 0.19-fold, p=0.0423). Finally, a significant correlation was found between the expression of PTHrP receptor and bcl-2 in cultured microvascular endothelial cells in the presence or absence of nicotine (Fig. 4C). These findings let us investigate whether the nicotine-dependent down-regulation of PTHrP receptors is also an α 7-dependent effect and whether it also depends on p38 MAP kinase activation. The effect of nicotine on PTHrP receptor expression was attenuated in the

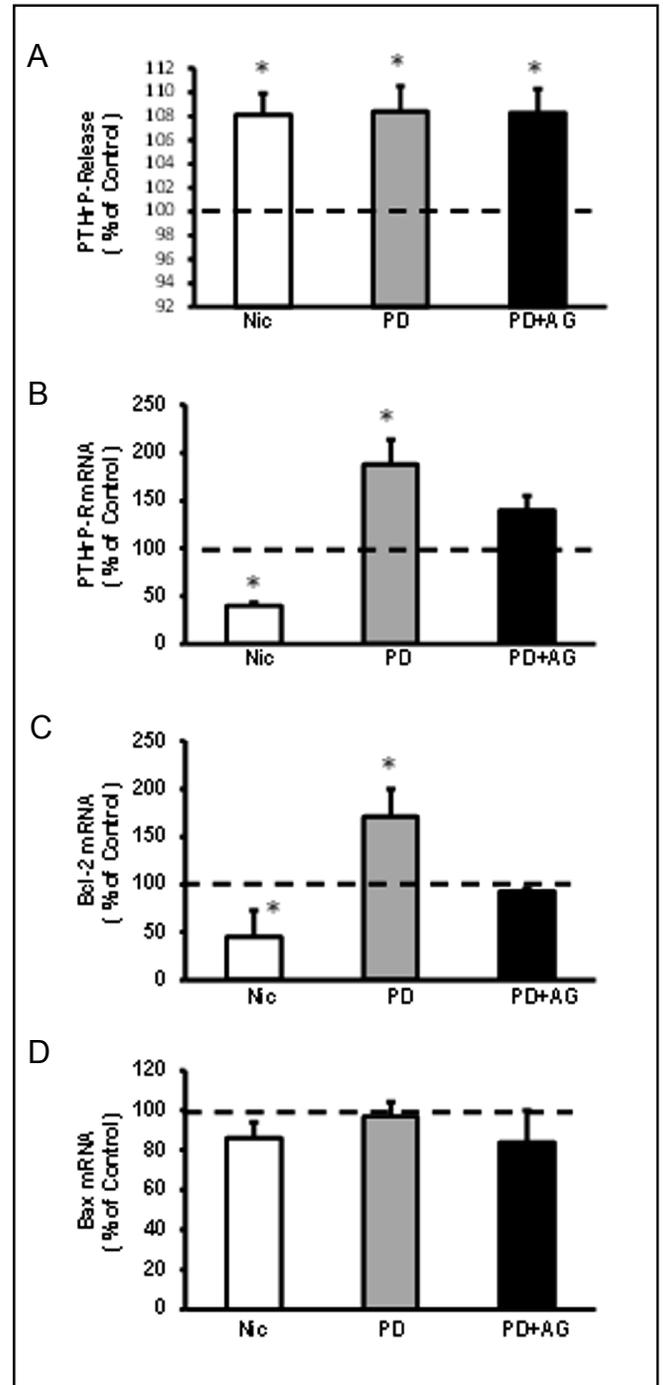


Fig. 6. Effect of nicotine and growth arrest on PTHrP release and subsequent change in expression of PTHrP receptor and apoptosis-related genes. A) PTHrP release (quantification of PTHrP in the supernatant of cultured endothelial cells). B) Effect on PTHrP receptor mRNA expression. C) Effect on bcl-2 expression. D) Effect on bax expression. Cells were cultured under control conditions, in the presence of nicotine (1 μ M), in the presence of an ERK activation inhibitor (PD98059, 10 μ M), and PD98059 and PTHrP(7-34, 1 μ M) to antagonize PTHrP receptor binding. Controls (dashed lines indicate PTHrP(7-34) alone). Data are means \pm S.D. from n=4 cultures. *,p<0.05 vs. control.

presence of the $\alpha 7$ -specific inhibitor bungarotoxin (Fig. 5). It was not affected by inhibition of p38 MAP kinase activation. Nicotine reduced PTHrP receptor expression by approximately 38% in the co-presence of a p38 MAP kinase activation inhibitor (Fig. 5).

Autocrine regulation of bcl-2 mRNA expression via PTHrP in microvascular endothelial cells

Our finding that nicotine down-regulates endothelial PTHrP receptors suggests that PTHrP controls bcl-2 mRNA expression in endothelial cells in an autocrine way. This hypothesis was therefore tested directly in the final experimental approach. Growth inhibition of microvascular endothelial cells increased the release of PTHrP from microvascular endothelial cells (Fig. 6A). Similarly, nicotine (1 μM) initiated an approximately 8% increase in extracellular PTHrP concentration. Growth inhibition was achieved by inhibiting the ERK activation pathway as used before [10]. Growth inhibition increased receptor expression and bcl-2 expression (Figs. 6B, C). As expected, the presence of a PTHrP receptor antagonist (PTHrP(7-34)) alone did not attenuate PTHrP release. However, in the presence of the PTHrP receptor antagonist, growth inhibition did no longer increase PTHrP receptor mRNA expression nor did it increase bcl-2 expression (Figs. 6B, C). In contrast, growth inhibition did not change the expression of bax (Fig. 6D). These data argue to an autocrine anti-apoptotic effect of PTHrP that seems to be avoid at high nicotine concentrations due to receptor down-regulation.

Effect of nicotine on endothelial cell apoptosis

We finally analyzed whether nicotine at a micromolar concentration, sufficient to down-regulate PTHrP receptors, induces apoptosis irrespectively of the corresponding up-regulation of the anti-apoptotic PTHrP. The number of apoptotic and necrotic cells was higher in nicotine treated cultures compared to untreated control cultures (Figs. 7A, B). Furthermore, nicotine decreased the mRNA expression of bcl-2 (Fig. 7C). No changes were observed in the expression of bax (Fig. 7C).

Discussion

This study was aimed to investigate the role of nicotinic acetylcholine receptor stimulation on PTHrP and PTHrP receptor expression in coronary endothelial cells. The results of the present study suggest that stimulation of nicotinic $\alpha 7$ receptors increases PTHrP protein

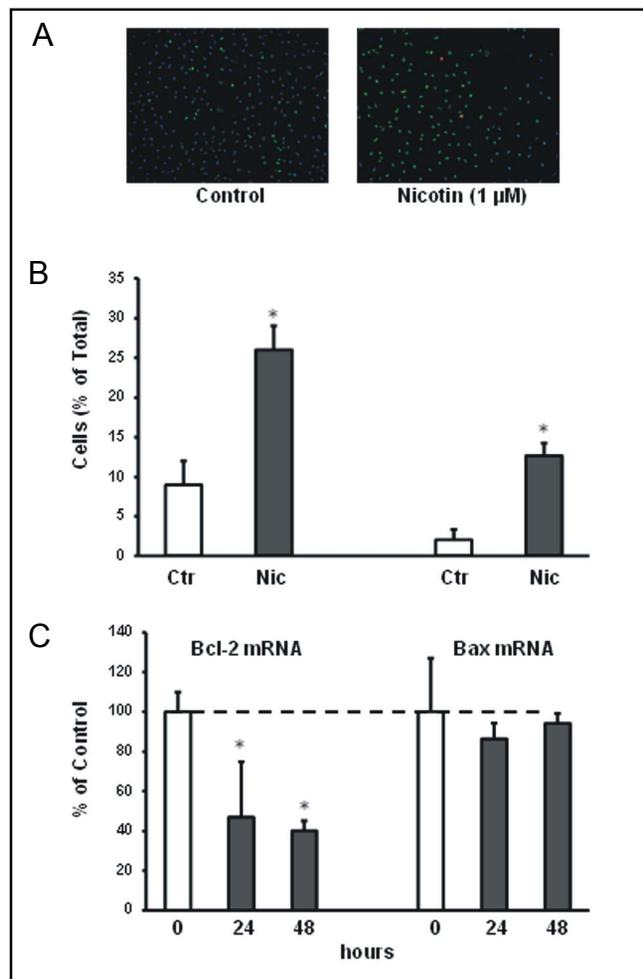


Fig. 7. Effect of nicotine (1 μM , 48 h) on apoptosis and necrosis. A) Representative fluorescence picture of endothelial cell cultures with normal nuclei (blue), apoptotic nuclei (green), and necrotic cells (red). B) Quantitative analysis of apoptotic (left) and necrotic (right) cells under control conditions and in the presence of nicotine (1 μM) (more than 2,600 cells each). C) Effect of nicotine (1 μM) on bcl-2 and bax expression (n=4). Data are means \pm S.D.; *, p<0.05 vs. control.

expression in these cells via an activation of the p38 MAP kinase pathway. PTHrP is known to protect endothelial cells against apoptosis via regulation of bcl-2 expression [18]. Here we show that this process requires the autocrine release of PTHrP. However, excessive nicotine concentrations are pro-apoptotic to these cells because they cause a down-regulation of PTHrP receptors and thereby attenuate the autocrine anti-apoptotic pathway.

Expression of nicotinic acetylcholine receptors on endothelial cells has been shown before. From the nine different α subunits ($\alpha 2$ - $\alpha 10$) the $\alpha 7$ subunit is the one that is most commonly be distributed in the vascular bed [19]. In human umbilical vein endothelial cells (HUVECs),

nicotinic acetylcholine $\alpha 7$ receptors are responsible for nicotine-dependent induction of the adhesion markers VCAM-1 and E-selectin [3]. Interestingly, $\alpha 7$ subunit-containing nicotinic acetylcholine receptors are characterized by a high Ca^{2+} permeability [20] and nicotine increases intracellular Ca^{2+} in HUVECs [3]. Here we show firstly that nicotine increases PTHrP expression in the low and nanomolar range. The concentration at which nicotine induced an effect on PTHrP protein expression was similar to that by which nicotine caused a decrease in BK_{Ca} and K_{ir} activity in HUVECs [4, 5]. Both responses on ion channels were again $\alpha 7$ subunit dependent. This is consistent with former reports about the role of $\alpha 7$ nicotinic acetylcholine receptors [21]. We showed before that release of PTHrP from microvascular endothelial cells depends on intracellular calcium changes as well [22]. Altogether, these results indicate the $\alpha 7$ subunits as the main important nicotinic acetylcholine receptor subunit in endothelial cells that contributes the regulation of PTHrP protein expression and release.

Intracellular signal transduction pathways activated by $\alpha 7$ subunits of nicotinic receptors have been linked to an activation of MAP kinase pathways in different cells types. In HUVECs and cultured microglial cells nicotine activates ERK and p38 MAP kinase pathways [3, 23]. Nicotine activates p38 MAP kinase on monocytes [24] but nicotine signalling in human lung cancer cells appears to require ERK activation and not p38 MAP kinase activation [25]. In our study we confirmed nicotine-dependent p38 MAP kinase signalling via $\alpha 7$ subunits in microvascular endothelial cells of the rat. This finding is consistent with some of the former reports. We exclude a participation of ERK and c-jun kinase activation in the PTHrP-specific response of endothelial cells to nicotine, consistent with a more cell type specific activation of the MAP kinase activation by nicotine. However, we also show that the corresponding down-regulation of PTHrP receptors does not depend on p38 MAP kinase activation.

Nicotine caused a concentration-dependent effect on PTHrP protein expression in microvascular endothelial cells. Similar to the bell-shaped concentration response curve found here, nicotine stimulates DNA synthesis and proliferation in vascular endothelial cells *in vitro* at low concentrations but shows cytotoxicity at higher concentrations [1]. The data of the current study suggest a positive feedback effect of PTHrP on its own receptor expression and regulation of bcl-2 expression via this autocrine loop. However, this positive feedback is disrupted at higher concentrations of nicotine. In our study

we found that nicotine directly reduces the expression of PTHrP receptor in endothelial cells. Furthermore, we show that nicotine reduces cardiac PTHrP receptor expression *in vivo*. Similar to our results, a nicotine-dependent decrease of PTHrP receptor binding has been reported for interstitial lipofibroblasts in the lung [12]. It is likely to assume that this latter effect of effect is also due to receptor down-regulation as recently been shown before [26, 27].

We confirmed a suggested relationship between PTHrP release, PTHrP receptor expression and bcl-2 regulation in a more direct way. Growth inhibition of endothelial cells leads to translocation of PTHrP into the nucleolus [10] and to a constitutive release of PTHrP [10]. In contrast to nicotine treated cells, endothelial cells with growth inhibition, performed by inhibition of the ERK pathway in this study, displayed an increased PTHrP release together with a higher PTHrP receptor type 1 expression. Under these conditions bcl-2 expression increased, too. As expected from these findings, addition of a PTHrP receptor antagonist attenuated the growth inhibition-dependent up-regulation of bcl-2 but not that of PTHrP release. These data show that PTHrP protects endothelial cells in an autocrine way. The finding is in agreement with data on chondrocytes but in apparent contrast to previous studies on MCF-7 breast cancer cells in which PTHrP protects against apoptosis via nuclear translocation [28, 29]. We provided some evidence in an earlier report that microvascular endothelial cells are also able to allow nuclear transport of PTHrP [10]. However, in the light of the present study an autocrine rather than an intracrine mechanism is more likely to be involved in the anti-apoptotic effect of PTHrP on endothelial cells. In addition, we found PTHrP rarely located into the nucleus in intact rat vessels (data not shown).

In conclusion, the current study provides evidence for deregulation of PTHrP-dependent control of bcl-2 expression and therefore loss of protection against apoptosis in endothelial cells exposed to excessive nicotine. This study adds another piece to the understanding of nicotine-dependent endothelial cell damage. The data indicate mechanisms by which nicotine, a major component of cigarettes, contributes to smoking-dependent vascular damage. Future studies are required to decide whether selective cholinergic receptor stimulation is able to increase PTHrP expression without a down-regulation of the corresponding receptor. In the light of the protective effects of PTHrP on endothelial cells this would be of importance.

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