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Evidence for Operation of Nicotinic and Muscarinic Acetylcholine Receptor-Dependent Survival Pathways in Human Coronary Artery Endothelial Cells

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ABSTRACT

Nicotinic acetylcholine receptors (nAChRs) have recently emerged as critical players in modulation of endothelial function. In particular, studies on endothelial cells from different vascular beds have shown anti-apoptotic actions of nicotinic stimulation, but whether there is actually activation of survival signaling downstream nAChR function has not been explored. In the present work we used human coronary artery endothelial cells (HCAECs) and a pharmacological approach to examine the impact of cholinergic stimulation on survival signaling pathways. Our findings show that cholinergic receptors promote activation of three typical survival routes: the phosphatidyl-inositol-3-kinase (PI3K)/AKT axis, activated downstream muscarinic and nAChRs; the JAK2/STAT3 axis, activated downstream nAChR; and ERK1/2 MAP kinases, activated by both muscarinic acetylcholine receptor (mAChR) and nAChR. Based on their sensitivity to α-bungarotoxin, nicotinic regulation of JAK2/STAT3 and ERK1/2 occurs downstream α7-nAChRs. The present findings suggest that in HCAECs the two cholinergic receptors may act concertedly to induce an efficient survival response of coronary cells when exposed to pro-apoptotic stimuli. J. Cell. Biochem. 112: 1978–1984, 2011.

KEY WORDS: CHOLINERGIC RECEPTORS; SURVIVAL SIGNALING; ENDOTHELIUM; APOPTOSIS; CORONARY ARTERY DISEASE

Over the last decade we have witnessed a significant progress in our knowledge of physiological and pathophysiological processes that are modulated by nicotinic acetylcholine receptors (nAChR) in non-neuronal tissues and organ systems. Indeed, many of the nAChR subtypes identified in neurons are also expressed in non-neuronal cells [Wessler and Kirkpatrick, 2008]. Among those is vascular endothelium, which exhibits a variety of functions that are, directly or indirectly, influenced by nicotinic cholinergic signaling, such as proliferation, migration, permeability changes in response to pro-inflammatory cytokines, and angiogenesis, among others [Cooke and Ghebremariam, 2008; de Jonge and Ulloa, 2007]. Importantly, nAChR function is emerging as a critical modulator of cellular and molecular mechanisms linked to the pathogenesis of inflammatory vascular disease, such as atherosclerosis. Recent studies indeed indicate that nAChR function may play a role in atherogenesis by modulating plaque neovascularization [Zhang et al., 2011], oxidative and phagocytic activity of macrophages [Wilund et al., 2009] and the pro-inflammatory and pro-apoptotic actions of cytokines on endothelial cells [Hakki et al., 2002]. The latter is of particular relevance to atherogenesis, as endothelial cell apoptosis is determinant in progression and fate of the lesion, particularly in advanced stages where the integrity of the endothelium covering or surrounding the plaque can be a defining factor to plaque stability and the precipitation of an acute coronary syndrome [Virmani et al., 2006]. It is thus of central importance to improve our understanding of the mechanisms underlying endothelial survival in order to identify components that might eventually be targeted to manipulate the endothelial cell's life at the lesion site. Here we report that in human coronary artery endothelial cells (HCAECs) cholinergic stimulation results in activation of three major signaling limbs associated to survival: a phosphatidyl-inositol-3-kinase (PI3K)/AKT axis downstream stimulation of

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muscarinic acetylcholine receptors (mAChRs) and nAChRs, nAChR-dependent activation of JAK2/STAT3 axis, and nicotinic and muscarinic-dependent stimulation of ERK1/2 MAP kinases. These findings suggest that in HCAECs nicotinic and muscarinic cholinergic signaling may act in a concerted manner to promote an efficient survival response of the coronary endothelium when cells are exposed to pro-apoptotic stimuli.

MATERIALS AND METHODS

Cell culture, immunoblotting, and terminal deoxynucleotidyl-transferase-dUTP-nick end labeling (TUNEL) assay were essentially as described by us [Smedlund et al., 2010; Smedlund and Vazquez, 2008; Tano and Vazquez, 2011]. Protocol details and antibody sources are provided in the online supplementary material.

RESULTS

In several cell types, including endothelial cells, activation of the phosphatidylinositol-3-kinase (PI3K)/AKT axis plays a key role in cell survival [Amaravadi and Thompson, 2005]. Based on existing evidence indicating an anti-inflammatory role of nAChRs and considering that those receptors are expressed in endothelial cells from diverse vascular beds [Cooke and Ghebremariam, 2008] we examined whether stimulation of nAChRs in HCAECs exerts a modulatory role on survival signaling. We first used the cholinomimetic agonist carbachol (CCh), which stimulates both mAChR and nAChRs and thus better resembles the physiological scenario, in which the two receptor pathways would be activated by their natural ligand acetylcholine. As shown in Figure 1A, CCh (100 μM) induced a time-dependent activation of AKT, as indicated by the extent of phosphorylation of AKT on Ser473. AKT phosphorylation occurred as early as 5 min, peaked at 10–15 min (four to fivefold over control) and declined to pre-stimulation levels after 30–60 min (see Supplementary Fig. IA for densitometric analysis). When cells were pre-treated with the mAChR antagonist atropine (1 μM) the effects of CCh were drastically reduced, particularly at the 10–15 min time points (Fig. 1B and Supplementary Fig. IB), while AKT phosphorylation was still noticeable at later times (30–60 min). This indicated a priori that both mAChR and nAChR contributed, although to different extents and with a different temporal profile, to AKT activation in HCAECs (see Discussion Section). To specifically examine the involvement of nAChR, we next exposed HCAECs to nicotine (10 μM). Interestingly, this resulted in biphasic AKT phosphorylation, with a first peak at 5 min (~2–3-fold over control) and a delayed effect at ~60 min (Fig. 1C and Supplementary Fig. IC), reflecting a pattern similar to that observed with CCh plus atropine. These actions of nicotine were almost completely reduced by the nAChR antagonist d-tubocurarine (dTC, 100 μM; Fig. 1D and Supplementary Fig. ID), confirming that the atropine-insensitive activation of AKT that followed CCh treatment occurred downstream nAChR. Pre-treatment of HCAECs with the PI3K inhibitor LY294002 (10 μM) abrogated cholinergic-induced AKT phosphorylation (not shown) indicating that activation of AKT took place downstream PI3K. A major mechanism of AKT-

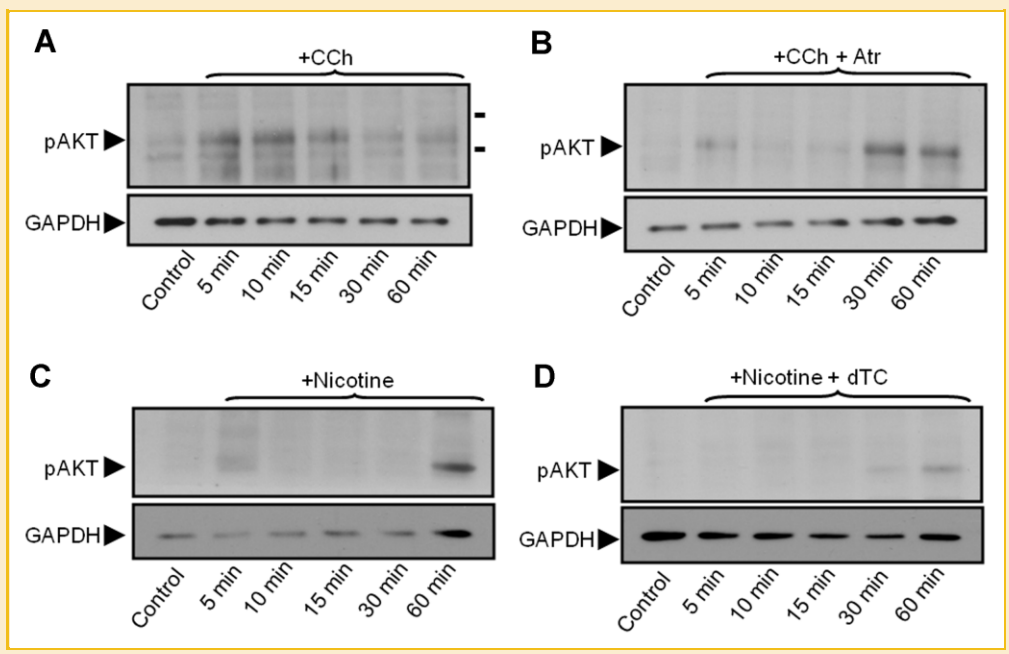

Fig. 1. HCAECs were treated with carbachol (CCh, 100 μM, A and B) or nicotine (10 μM, C and D) for the indicated times in the absence (A and C) or presence of atropine ("Atr," 1 μM, B) or d-tubocurarine ("dTC," 100 μM, D). In all instances, cells were processed for immunodetection of phospho-AKT (Ser473; 60 kDa) in whole cell lysates. When indicated, atropine and dTC were added 15 min before stimulation and kept throughout the entire duration of the experiment. Membranes were reprobed for GAPDH (37 kDa) to control for protein loading. Blots are representative from at least three independent experiments. In A, positions of the 52 and 76 kDa molecular weight markers are indicated on right side of the blot for reference.

dependent cell survival operates through AKT-mediated phosphorylation of its downstream target BAD, a pro-apoptotic Bcl-2 family member, on Ser136 [Amaravadi and Thompson, 2005]. Supplemental Figure II shows that CCh induced atropine-sensitive increase in phosphorylation of BAD (Ser136) which correlated well with the CCh-dependent activation of AKT. Nicotine treatment did not affect BAD phosphorylation (not shown).

In cell types other than endothelial, for example, neurons and macrophages ([Marrero et al., 2011] and references therein) activation of STAT3 and the MAP family kinases ERK1/2 has been shown to occur downstream nAChR stimulation and represents an alternative pro-survival pathway to the PI3K/AKT axis. Notably, CCh treatment of HCAECs resulted in time-dependent activation of STAT3, as indicated by an increase in the extent of STAT3 phosphorylation on Tyr705 (Fig. 2A), which peaked around 5–10 min (~4–5-fold increase over control, see Supplementary Fig. IIIA) and rapidly declined to pre-stimulation levels. CCh-induced STAT3 phosphorylation was insensitive to atropine (Fig. 2B and Supplementary Fig. IIIB) suggesting stimulation downstream nAChRs. In line with this notion, levels of phospho (Tyr705)-STAT3 were significantly elevated following treatment with nicotine, and this effect was fully prevented by dTC (Fig. 2C and Supplementary Fig. IIIC). Nicotine-induced activation of STAT3 was markedly diminished by pre-treating cells with the JAK2 selective inhibitor AG490 (10 μM, Fig. 2C) suggesting operation of a nAChR/JAK2/STAT3 axis. In line with this, HCAECs treated with nicotine exhibited a time-dependent increase in JAK2 (Tyr221) phosphorylation that was abrogated by AG490 (Supplementary Fig. IVA–C). Notably, nicotine-dependent phosphorylation of JAK2 and STAT3 was suppressed by the α7-nAChR selective blocker α-bungarotoxin (100 nM; Fig. 2C and Supplementary Fig. IVD).

CCh treatment of HCAECs also resulted in time-dependent activation of ERK1/2 MAP kinases, manifested as an increase in ERK1/2 phosphorylation (Thr202/Tyr204 of ERK1, Thr185/Tyr187 of ERK2) that peaked at 5 min following cholinergic stimulation (Fig. 3A). This effect was partially decreased by atropine (~50% reduction; Supplementary Fig. VA) indicating nicotinic and muscarinic cholinergic contribution to ERK1/2 stimulation. In line with this, nicotine treatment also augmented the levels of phospho-ERK1/2 in a dTC-sensitive manner (Fig. 3B and Supplementary Fig. VB). Remarkably, nicotinic activation of ERK1/2 was completely prevented by α-bungarotoxin (100 nM; Fig. 3C) suggesting involvement of α7-nAChRs. In agreement with this interpretation, challenging HCAECs with the α7-nAChR selective agonist GTS-21 (30 μM; [Kem, 1997; Nai et al., 2003]) promoted ERK1/2 phosphorylation—although somewhat delayed compared to nicotine- in an α-bungarotoxin-sensitive manner (Supplementary Fig. VI). We recently showed that in HCAECs pro-inflammatory actions of the atherorelevant cytokine TNFα causes rapid (within 5 min) and sustained (up to 1 h) activation of NFκB, based on the extent of IκBα phosporylation/degradation ([Smedlund et al., 2010], and Supplementary Fig. VII). Moreover, it has been shown that cholinergic stimulation can prevent endothelial cell activation and leukocyte recruitment through inhibition of NFκB [Saeed et al., 2005]. When HCAECs were challenged with carbachol (100 μM) or nicotine (10 μM) no significant changes were observed in IkBα

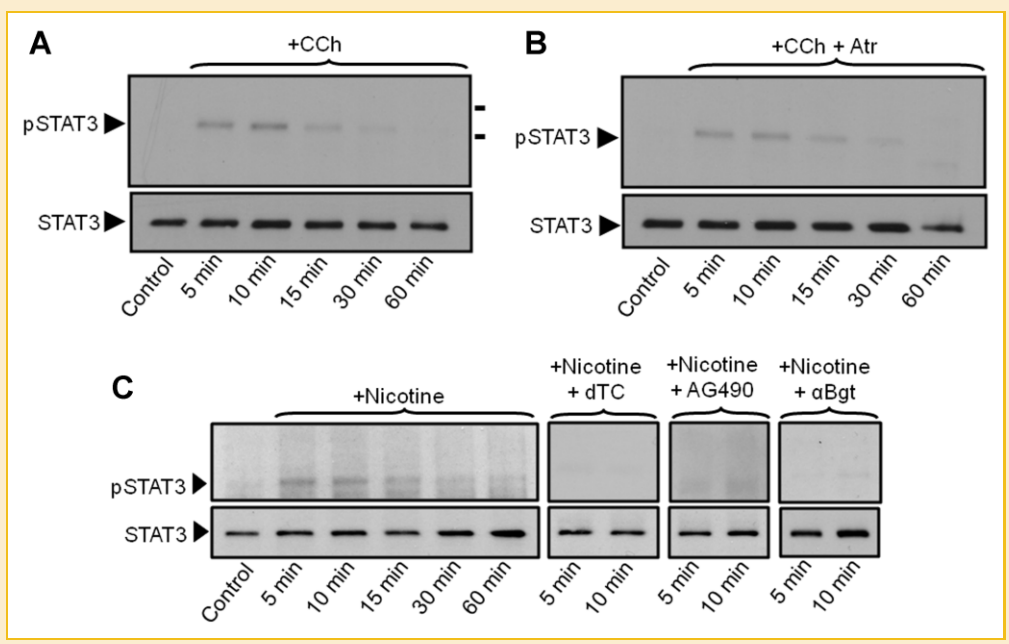

Fig. 2. HCAECs were treated with carbachol (CCh, 100 μM) for the indicated times in the absence (A) or presence (B) of atropine ("Atr," 1 μM) and then processed for immunodetection of phospho-STAT3 (Tyr705; ~80 kDa) in whole cell lysates. Alternatively, cells were exposed to nicotine (10 μM, C) for the indicated times in the absence or presence of d-tubocurarine ("dTC," 100 μM), AG490 (10 μM) or α-bungarotoxin ("αBgt," 100 nM, 15 min pre-incubation) before immunodetection of phospho-STAT3 (Tyr705). When indicated, dTC and AG490 were added 15 min before stimulation. Membranes were reprobed for total STAT3 to control for protein loading. Blots are representative from at least three independent experiments. In A, positions of the 76 and 102 kDa molecular weight markers are indicated on right side of the blot for reference.

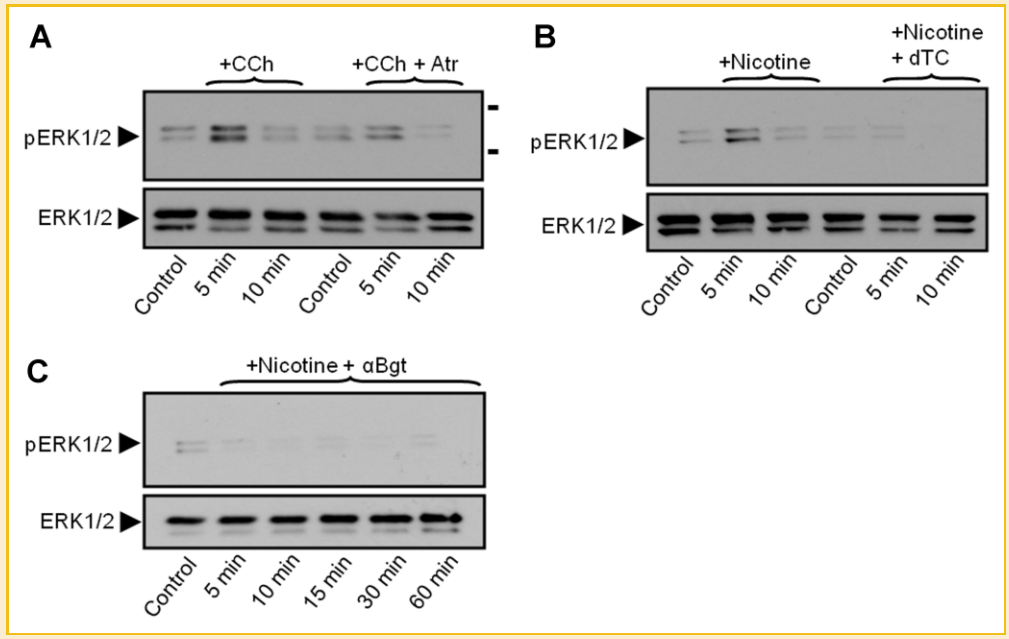

Fig. 3. A: HCAECs were treated with carbachol (CCh, 100 μM) for the indicated times in the absence or presence of atropine ("Atr," 1 μM, 15 min pre-treatment) and then processed for immunodetection of phospho-ERK1/2 (Thr202/Tyr204 of ERK1, Thr185/Tyr187 of ERK2; 42/44 kDa) in whole cell lysates. Alternatively, cells were exposed to nicotine (10 μM, B) for the indicated times in the absence or presence of d-tubocurarine ("dTC," 100 μM, 15 min pre-treatment) or (C) α-bungarotoxin ("αBgt," 100 nM, 15 min pre-incubation), as indicated, before immunodetection of phospho-ERK1/2. Membranes were reprobed for total ERK1/2 to control for protein loading. Blots are representative from at least three independent experiments. In A, positions of the 38 and 52 kDa molecular weight markers are indicated on right side of the blot for reference.

phosphorylation or degradation, nor in the ability of TNFα (10 ng/ml) to activate NFκB (Supplementary Fig. VII). Also, cholinergic stimulation of HCAECs failed to induce VCAM-1 expression (not shown) in line with our previous finding that in these cells VCAM-1 is under complete control of NFκB [Smedlund et al., 2010].

Finally, we wanted to explore if stimulation of cholinergic receptors had an impact on the rate of apoptosis of HCAECs. To answer this important question we examined TNFα-induced apoptosis in HCAECs in the absence or presence of cholinergic stimulation, by using a TUNEL assay. HCAECs were incubated for 48 h in endothelial basal medium with or without (control) TNFα (10 ng/ml), and with or without concomitant incubation with muscarinic or nAChR agonists and/or antagonists. As expected, TNFα treatment resulted in appearance of a significant number of apoptotic cells (~4-fold increase in TUNEL-positive cells) compared to control (Fig. 4). Cholinergic agonists or antagonists did not induce apoptosis when added alone to the medium. However, simultaneous incubation of cells with TNFα in the presence of nicotine or CCh, resulted in a statistically significant reduction of the number of TUNEL-positive cells compared to cells treated with the cytokine alone. Notably, blocking mAChR or nAChR completely prevented the protective actions of the cholinergic agonists.

DISCUSSION

The present work provides for the first time evidence showing that stimulation of nicotinic and mAChRs triggers pro-survival signaling in endothelial cells derived from human coronary arteries (HCAECs). When the cholinomimetic carbachol was used to activate nAChR and mAChR, three survival signaling branches were activated: PI3K/AKT, JAK2/STAT3, and the MAPK family members ERK1/2 (see model, Supplementary Fig. VIII). An immediate speculation can be made, and is that under physiological conditions of receptor stimulation with acetylcholine as the endogenous ligand, concomitant cholinergic-dependent activation of those signaling pathways likely accounts for an efficient survival response of coronary endothelial cells when exposed to an inflammatory setting.

Our results show that whereas both mAChR and nAChR activated PI3K/AKT in HCAECs, nicotinic stimulation of AKT exhibited a biphasic temporal course compared to a monophasic effect of muscarinic signaling. One interpretation is that muscarinic stimulation also triggers compensatory mechanisms that turn off AKT—not unusual in G protein coupled receptor modulation of AKT [Liu et al., 2004]; as stimulation persists, deactivation takes over and masks the delayed nicotinic signaling when both mAChR and nAChR are simultaneously activated. Despite these differences, the characteristics of mAChR and nAChR stimulation of AKT were highly comparable to that of other well known activators of AKT in the coronary circulation [Erdogdu et al., 2010; Mahadev et al., 2008; Teng et al., 2011]. In several cell types activation of AKT by muscarinic receptors is subsequent to direct actions of either $G_{\alpha i}$- or $G_{\alpha q}$-depending on the type of mAChR- and $G_{\beta\gamma}$ on both PI3K and AKT [Resende and Adhikari, 2009]. As for the nAChR, in rat pheochromocytoma PC12 cells nicotine-dependent stimulation of PI3K/AKT occurs downstream JAK2, and involves direct interaction between α7-nAChR and JAK2; this is followed by tyrosine

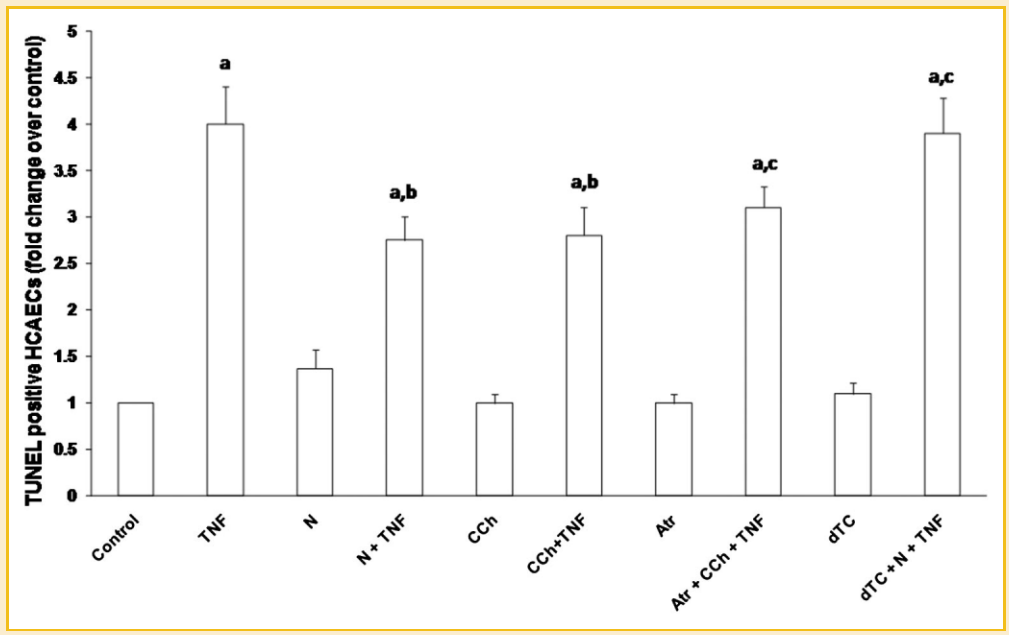

Fig. 4. HCAECs were grown on 24-well plates and exposed for 48 h (37°C in humidified air/5% CO_2 atmosphere) to endothelial basal medium in the absence ("Control") or presence of TNFα ("TNF," 10 ng/ml), nicotine ("N," 10 μM), carbachol ("CCh," 100 μM), atropine ("Atr," 1 μM) or d-tubocurarine ("dTC," 100 μM), or the indicated combinations. Following treatments HCAECs were processed for evaluation of apoptosis by TUNEL assay, as described in Materials and Methods Section. $^{a}P < 0.01$ respect to "Control"; $^{b}P < 0.05$ respect to "TNF" alone; $^{c}P < 0.05$ respect to "N + TNF" or "CCh + TNF." For each experimental condition the change in number of apoptotic (TUNEL-positive) cells was normalized to the respective control and results expressed as fold respect to control.

phosphorylation of JAK2, activation of PI3K, and recruitment and activation of AKT [Shaw et al., 2002]. In non-neuronal cell types, such as macrophages or microglia, nAChR activation of PI3K/AKT seems to be Ca^{2+}-dependent, but is not clear if this is subsequent to ion channel Ca^{2+} fluxes or related to nAChR-dependent activation of PLC and Ca^{2+} release from stores ([de Jonge and Ulloa, 2007] and references therein). Molecular and pharmacological studies will be required to determine how in HCAECs activation of mAChR or nAChR couples to activation of PI3K/AKT.

In macrophages, cholinergic activation of the JAK2/STAT3 pathway takes place downstream α7-nAChR [Marrero et al., 2011]. Our observation that nicotine-induced phosphorylation of JAK2 and STAT3 are inhibited by α-bungarotoxin, represent the first evidence to date indicating activation of the JAK2/STAT3 axis downstream α7-nAChRs in human coronary endothelial cells. Unlike our findings in HCAECs, recent work showed that nicotine treatment of human umbilical vein and dermal microvascular endothelial cells inhibits JAK2/STAT3 [Chatterjee et al., 2009]. Rather than contradictory, these findings likely reflect heterogeneity of endothelial cells derived from different vascular beds in regards to their responsiveness to cholinergic stimulation.

Similar to the PI3K/AKT axis, cholinergic activation of ERK1/2 in HCAECs is contributed by both mAChR and nAChR. Notably, nicotinic stimulation of ERK1/2 was also completely abolished by α-bungarotoxin, again indicating involvement of α7-nAChRs. Interestingly, growth factor and activity-dependent survival of chick ciliary ganglion neurons is promoted by α7-nAChRs, at least in part, through ERK1/2-mediated signaling [Pugh and Margiotta, 2000, 2006; Pugh et al., 2006]. It is thus tempting to speculate that α7-nAChR-dependent modulation of JAK2/STAT3 and ERK1/2 pathways represents a general mechanism of nicotinic cholinergic regulation of survival in different cell types. In the case of HCAECs, the question arises as to how persistent activation of nAChRs, as expected to occur in smokers which have sustained circulating levels of nicotine, affects the balance of mAChR versus nAChR survival signaling in the context of coronary pathology.

Importantly, the present studies also show a *bona fide* anti-apoptotic effect of sustained cholinergic stimulation when HCAECs were exposed to inflammatory conditions. In HCAECs nicotine has been shown to reduce apoptosis induced by combined treatment with TNFα and dexamethasone [Hakki et al., 2002]. However, in these cells dexamethasone interferes with inflammatory signaling [Zouki et al., 2000] making it difficult to conclude if those results were subsequent to a genuine anti-apoptotic action of nicotine rather than a combined action of nicotinic and glucocorticoid signaling on expression/function of pro-apoptotic molecules. Our findings showing lack of cholinergic activation of the NFκB pathway or VCAM-1 induction in HCAECs are in line with the notion of a pro-survival, protective rather than pro-inflammatory role of cholinergic signaling in these cells. In the particular case of nAChR signaling in endothelial cells from coronary circulation, studies aimed at evaluating nicotine effects have yielded rather controversial results. For example, the expression of VCAM-1 and ICAM-1, key mediators of monocyte recruitment, was shown to be augmented by nicotine treatment of HCAECs in one study [Cirillo et al., 2007] while work from other laboratory showed no effect [Hakki et al., 2002]. Whereas these controversial findings may be partly due to differences in experimental conditions, it is also

possible that the high membrane permeability of nicotine may induce effects beyond those strictly related to its actions on nAChRs, especially during prolonged incubation times with the alkaloid. Within this context, by showing equivalent outcomes when stimulating nAChR with either nicotine or carbachol—but blocking mAChR signaling with atropine our findings strongly indicate operation of a genuine nicotinic cholinergic effect on survival signaling rather than non-specific effects of nicotine.

We have not been able to detect cholinergic-induced Ca^{2+} influx in HCAECs by means of conventional Fura-2-based Ca^{2+} imaging techniques (not shown). However, these cells have a very efficient Ca^{2+} buffering system [Smedlund and Vazquez, 2008] and thus the possibility exists that Ca^{2+} entering through nAChRs is rapidly coped by the buffering apparatus and goes undetectable. Using rat coronary microvascular endothelial cells Adams and colleagues [Moccia et al., 2004] showed the existence of functional nAChRs which mediate inward currents carried primarily by Na^{+}. Additional studies are needed to define if in HCAECs nAChRs exhibit similar permeability properties. This will also define whether the role of nAChR in survival signaling in HCAECs is related to channel function or if it merely reflects a signaling role independent of channeling properties, as shown in cell types other than endothelial [de Jonge and Ulloa, 2007]. Given the fundamental role of endothelial cell survival/apoptosis in atherogenesis, the present findings, although strictly pharmacological, represent a timely and novel contribution to our understanding of molecular events that might be of relevance for cholinergic-dependent modulation of coronary endothelial cell survival in the inflammatory setting of atherosclerosis.

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