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Functional role of nicotinic acetylcholine receptors in apoptosis in HL-60 cell line

Delphine Gimonet[a,1], Regis Grailhe[b,2], Paul Coninx[a,1], Frank Antonicelli[c,3], Bernard Haye[c,3], Françoise Liautaud-Roger[a,*]

[a] *Institut Jean-Godinot, Secteur Prévention, BP 171, 1 Avenue du général Koenig, 51056 Reims Cedex, France*
[b] *Laboratoire Récepteur et Cognition, Institut Pasteur, 25 rue du Docteur Roux, 75015 Paris, France*
[c] *FRE CNRS 2534, Faculté des Sciences, Université de Reims, Moulin de la Housse, BP 1039, 51687 Cedex 2, France*

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Abstract

The subunit composition of nicotinic acetylcholine receptors involved in apoptosis is an ongoing question. HL-60 cells were used in order to investigate the implication of nicotinic acetylcholine receptors in bleomycin-induced apoptosis. We found that bleomycin-induced apoptosis was significantly enhanced by nicotine and was blocked by nicotinic acetylcholine receptor antagonists, including α-bungarotoxin, a competitive antagonist of α7 nicotinic receptor. Among the other agonists tested, 3-[2,4-dimethoxybenzylidene]anabaseine (GTS-21)-selective agonist for α7-nicotinic acetylcholine receptor-, but not epibatidine or cytisine, enhanced bleomycin-induced apoptosis. In addition to these results, the detectable presence of α7-mRNA supports a key role of α7-nicotinic acetylcholine receptors in the modulation of the induced apoptosis by nicotine.

Keywords: Nicotinic receptor; α7-Subunit; Bleomycin; Apoptosis; HL-60 cell

1. Introduction

Nicotine, as a main component of cigarette smoke, affects the organism through nicotinic acetylcholine receptors. These receptors belong to the pentameric ligand-gated ion channels, which consist of 16 different subunits that form homo- and hetero-pentameric receptors (Lindstrom et al., 1998). In addition to the central nervous system, nicotine is known to affect many other tissues such as the respiratory tract, skin, hematological cells, vascular and immune tissues (Richman, 1979; Grando et al., 1995; Macklin et al., 1998; Maus et al., 1998; Sato et al., 1999). Of special interest is the role of nicotinic acetylcholine receptors in apoptosis phenomena related to nicotine. It is widely reported that the alkaloid produced a protective effect upon induced apoptosis (Wright et al., 1993; Li et al., 1999; Garrido et al., 2001; Hakki et al., 2001, 2002; Sugano et al., 2001) that could be consecutive to *Bcl-2* activation (Mai et al., 2003). Nevertheless, in a recent study, nicotine appeared to enhance the programmed cell death triggered by deoxycholate, a cytotoxic bile salt suspected to interfere in colon carcinogenesis (Crowley-Weber et al., 2003). The authors' model is rather close to the biological system used in the present study with nicotine administered at sub-micromolar level such as that observed in smokers.

The ubiquitous distribution of nicotinic acetylcholine receptors and their possible involvements in many medical fields, especially in cancer, prompt us to initiate investigations on apoptosis. Our model is based on the use of a radiomimetic agent bleomycin on HL-60 cell line known to be from hematological origin and likely to express nicotinic acetylcholine receptors (Richman, 1979; Lebargy et al., 1996; Benhammou et al., 2000; Villiger et al., 2002) together with the *bcl-2* anti-apoptotic gene (Han et al., 1995; Wen et al., 2000). The aim of the study was (i) to observe the nicotine effects on bleomycin-induced apoptosis

* Corresponding author. Tel.: +33-3-26-50-42-87; fax: +33-3-26-50-43-98.
E-mail addresses: rgrailhe@pasteur.fr (R. Grailhe), frank.antonicelli@univ-reims.fr (F. Antonicelli), francoise.roger@reims.fnclcc.fr (F. Liautaud-Roger).
[1] Tel.: +33-3-26-50-42-87; fax: +33-3-26-50-43-98.
[2] Tel./fax: +33-1-45-68-88-46.
[3] Tel.: +33-3-26-91-34-35; fax: +33-3-26-91-31-68.

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in HL-60 cell line, (ii) to determine whether nicotine acted through nicotinic acetylcholine receptors, and (iii) if so, to determine pharmacologically the subunit of the nicotinic acetylcholine receptors.

2. Materials and methods

2.1. Cell culture

Human promyelocytic leukemia cells (HL-60; American Type Culture Collection, Rockville, USA) were grown at 37 °C in RPMI 1640 medium (BioWhittaker, Bio-Sciences, Emerainville, France) supplemented with 20% fetal calf serum (v/v), 100 U/ml penicillin, 100 μg/ml streptomycin and 0.025 μg/ml amphotericin B (GIBCO-BRL, Grand Island, USA) in 5% CO_2 atmosphere. The cell density was re-adjusted to 2×10^5 cells/ml, every 2 days. Subcultures for pharmacological investigations were carried out from cells which were between the 4th and 40th passage. Viable cells were evaluated by trypan blue exclusion test.

2.2. Reverse transcription-polymerase chain reaction experiments

Total RNA was extracted by Tri-reagent method according to the manufacturer's instructions (Sigma, St. Louis, MO, USA). Samples (50 μg) were reverse-transcribed using M-MLV reverse transcriptase (GIBCO-BRL). Polymerase chain reaction (PCR) was run for 4 min at 95 °C and for 40 cycles at 95 °C for 15 s, at 55 °C (except for the β4 primers, which were annealed at 52 °C) for 15 s, at 72 °C for 45 s plus 7 min at 72 °C in a Perkin-Elmer 9600 thermal cycler. Reverse transcripts-PCR products were analysed by electrophoreses on a 2% agarose gel stained with ethidium bromide. A 100 base pair DNA Ladder (MBI Fermentas) was used as molecular weight standard. The primers were designed for optimal priming efficiency based on coding sequences available in GenBank. Their sequences and expected product size (in parentheses) are for α3, 5′-GCTGGTGAAGGTGGATGAAGTAAA-3′ and 3′-CTC-GCCGCTCTCCCAATAGTCC-5′ (409 base pair); for α4, 5′-TTCTCCGGTTACAACAAGTGGTC-3′ and 3′-CTGCTGGTCGAAGGGGAAGA-5′ (399 base pair); for α7, 5′-GGCCAATGACTCGCAACCACT-3′ and 3′-GAC-CAGCCTCCGTAAGACCAG-5′ (399 base pair); for β2, 5′-TGGGAAGATTATCGCCTCACCT-3′ and 3′-CAGCGC-CACGATGTCCCACTCACC-5′ (365 base pair); for β4, 5′-GCGCCTTCCCTGGTCCTTTTCTTC-3′ and 3′-TAGGTCCCGTCGGCGTTGTTGTAA-5′ (365 base pair); for β2-microglobulin, 5′-AGCAGAGAATCGAAAGT-CAAA-3′ and 3′-TGTTGATGTTGGATAAGAGAAT-5′ (532 base pair). Non-reverse-transcribed RNA were used as a template to control some eventual contaminations at every step of the procedure.

2.3. Apoptosis—induction and measurement

At day −1, HL-60 cells (4×10^5/ml) were grown with RPMI 1640 medium containing 2.5% fetal calf serum. At day 0, cell density was re-adjusted to 4×10^5/ml and cells were seeded in 48-well plates. The time of exposure to bleomycin (from Aventis, Aulnay sous Bois, France) and the experimental plan with nicotine were drawn from published data concerning bleomycin-induced karyotypic alterations (Cloos et al., 1996), smokers' behavior and their nicotine blood levels (Henningfield, 1995; Benowitz et al., 2001). Bleomycin was tested for various concentrations: 50, 100, 160 and 210 μM over a period of 7.5 h in order to determine the best ratio of apoptosis vs. necrosis, i.e. less than 1% of trypan blue cells. The quantitative evaluation of apoptosis is based on cells counted together with morphological features of apoptosis. Cell spots, obtained by cytocentrifugation (Cytospin, Shandon, Pittsburg, USA) for 5 min at $50 \times g$, were stained by GIEMSA solution (Merck, Darmstadt, Germany). The mean percentage of cells with a condensed or fragmented nucleus was derived after counting two times ≥ 500 cells in random microscopic fields. Results provided an intra-observer and intra-experiment reproducibility of 6% (i.e. the coefficient of variation).

2.4. Pharmacological investigations

The nicotinic acetylcholine receptor agonists and antagonists were obtained at Sigma, except for 3-[2,4-dimethoxybenzylidene]anabaseine (GTS-21), which was generously provided by W.R. Kem (Florida University, Gainsville). As agonists, (−)-nicotine tartrate ($n = 10$), (+/−)-epibatidine dihydrochloride ($n = 4$), (−)-cytisine ($n = 4$) and GTS-21 ($n = 4$) were used at concentrations of 6, 60, 600 and 6000 nM. As antagonists, mecamylamine hydrochloride was experienced at 10 μM ($n = 3$), D-tubocurarine chloride at 100 μM ($n = 3$) whereas α-bungarotoxin was tested at a wide range of concentrations from 0.1 nM to 1 μM ($n = 10$). Based on a cutoff value of 0.1 μM, two equivalent groups of data ($n = 5$) were subsequently made for statistical analyses. The experimental schedules will be specified in Figs. 2–4.

2.5. Statistical analysis

The agonist concentrations were considered to be ordered categories and the presence of antagonists was used as a binary variable (yes/no). In each pharmacological group of experiments, variations of apoptosis were analysed by analysis of variance under general linear model (SPSS10 software). Multiple comparisons between the apoptotic levels from each concentration of agonist versus that from control were carried out by post hoc bilateral Dunnett's *t*-tests for unpredictable effects and by unilateral tests for expected effects. The percentages of apoptotic cells were expressed as relative level of apoptosis which resulted from dividing each value by the mean percentage of the controls,

i.e. that corresponding with [nicotine] 0 nM or [agonists] 0 nM, respectively, in Figs. 2 and 3 and in Fig. 4.

3. Results

3.1. Nicotinic acetylcholine receptors

We analysed HL-60 cells by reverse transcriptase-PCR to determine which nicotinic acetylcholine receptor subunits are expressed. Transcripts coding for α7-, β2- and β4-subunits, α3- and α4-nicotinic acetylcholine receptor subunits were found to be expressed in HL-60 (Fig. 1). Thus, multiple subtypes of nicotinic acetylcholine receptors may be translated and play a functional role in regulating HL-60 cell activity.

3.2. Spontaneous apoptosis and effects of nicotine

In the absence of both nicotine and bleomycin, spontaneous apoptosis ranged from 0.4% to 6.4% with an asymmetric distribution around the median, at 1.5% ($n=21$). Among the agonists and the antagonists (data not shown), only nicotine displayed some effect on apoptosis basal level which was found to be reduced significantly to 68% (nicotine factor: $F(4,15)=8.1$; $P=0.001$; bilateral Dunnett's t-test significant at 6 nM; $P=0.002$) (Fig. 2, left).

3.3. Nicotine effect on bleomycin-induced apoptosis

The optimal dosage of bleomycin was established at 160 μM since it was found that for higher concentration (i.e. 210 μM) the necrosis level was superior to 1%. In the presence of bleomycin, the mean level of apoptosis was measured at 8.1% (95 CI 7.2–9.3; $n=23$). Nicotine increased 1.28-fold the bleomycin-induced apoptosis over the baseline level at 600 nM (nicotine factor: $F(4,45)=8.1$; $P<0.0001$; bilateral Dunnett's t-test significant at 600 nM, $P<0.0001$; $n=10$) (Fig. 2, right).

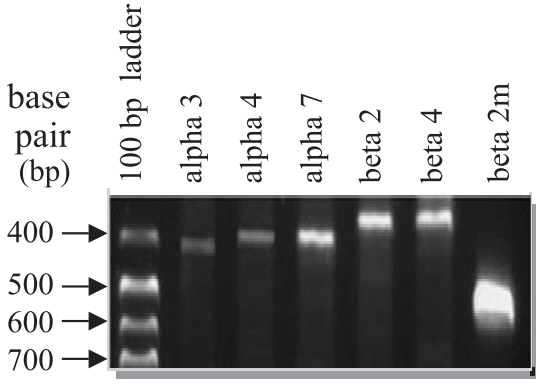

Fig. 1. Agarose gel electrophoresis of the reverse transcriptase-polymerase chain reaction products from total RNA of HL-60 cell line. Transcript of the α3- (409 base pair), α4- (399 base pair), α7- (399 base pair), β2- (365 base pair) and β4- (365 base pair) nicotinic acetylcholine receptor subunits were detected. β2-Microglobulin (β-2m) (532 base pair) was used as the positive control.

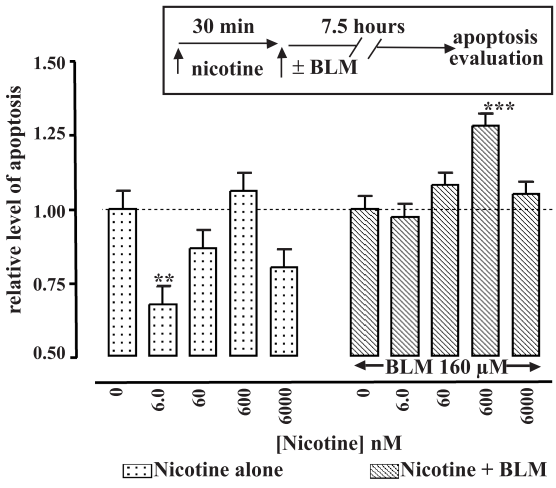

Fig. 2. Effect of increasing concentrations of nicotine (*X*-axis) on apoptosis in the absence (*Y*-axis; on the left side of the figure) and in the presence of bleomycin (BLM) (*Y*-axis; right side). In the absence of bleomycin, nicotine—at 6 nM—decreases apoptosis (**$P<0.01$) as opposed to an increase at 600 nM in presence of bleomycin (***$P<0.001$). However, the baseline normalized apoptosis in controls between either side of the figure corresponds to different levels of apoptotic cells (see Results). Error bars represent standard error of the mean. The insert at the top indicates the time course of the experiment.

3.4. Nicotinic receptor antagonists block the nicotine effect on bleomycin-induced apoptosis

As expected, there was an interaction between antagonists and nicotine (antagonist × nicotine: $F(12,110)=4.96$; $P<0.001$). Each antagonist blocked not only the increase of bleomycin-induced apoptosis of nicotine at concentration

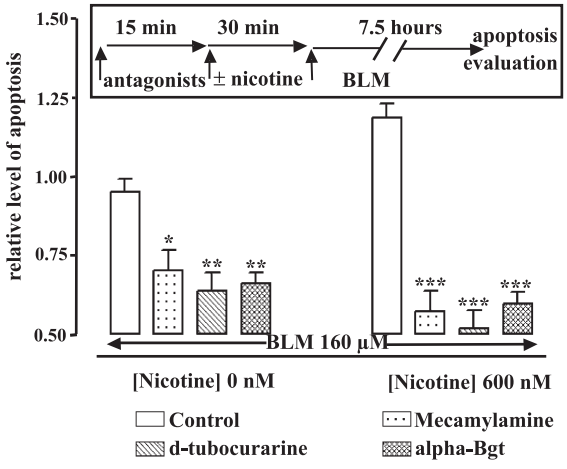

Fig. 3. Effect of three antagonists on bleomycin(BLM)-induced apoptosis (*Y*-axis) in the absence of nicotine (*X*-axis, left) and in the presence of nicotine at 600 nM (right). The antagonist effect is observed in both conditions (*$P<0.05$; **$P<0.001$ and ***$P<0.0001$). The insert at the top indicates the time course of the experiment. Error bars represent standard error mean (α-Bgt = α-bungarotoxine).

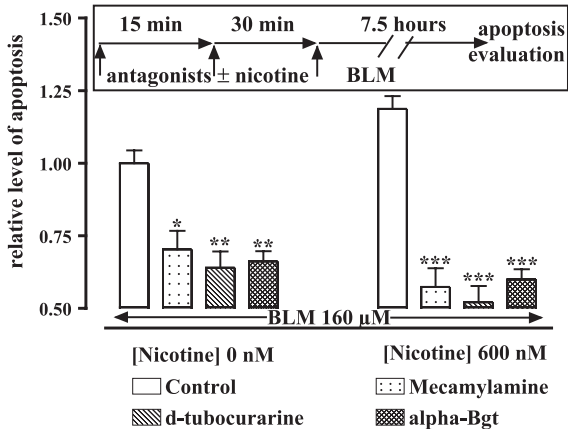

Fig. 4. Effect of increasing concentrations of three agonists (*X*-axis) on bleomycin(BLM)-induced apoptosis (*Y*-axis). Only the agonist specific for α7-nicotinic acetylcholine receptor (i.e. 3-[2,4-dimethoxybenzylidene]anabaseine (GTS-21)) produced an increase of apoptosis (**$P<0.01$). Error bars represent the 95 confidence interval. The insert at the top indicates the time course of the experiment.

600 nM but also lowered the baseline apoptosis to approximately 60% and, for α-bungarotoxin, independently of its concentrations either <1 or ≥ 0.1 μM. Furthermore, all antagonists exerted as well a protective effect against bleomycin-induced apoptosis independently of the presence of nicotine (Fig. 3). Therefore, all these data concerning the antagonists were merged for further analyses. The antagonist effect appeared as being significant within controls (antagonist within control: $F(1,124)=5.33$, $P=0.02$) and within the nicotine concentration 600 nM ($F(1,124)=21.0$, $P<0.0001$).

3.5. Pharmacological dissection of nicotinic acetylcholine receptors

Among the agonists, only GTS-21-specific for α7-nicotinic acetylcholine receptor-mimicked the nicotine effect on bleomycin-induced apoptosis with a significant increase at 60 nM (GTS-21 factor: $F(4,15)=6.45$; $P<0.03$; unilateral Dunnett's *t*-test significant at 60 nM: $P=0.003$). The apoptosis rose 1.29-fold beyond the baseline. In contrast, both the heteromeric nicotinic acetylcholine receptor agonists (cytisine and epibatidine) were ineffective on bleomycin-induced apoptosis (Fig. 4).

4. Discussion

The present study aimed to develop a model for investigating functional implication of nicotinic acetylcholine receptors under nicotine concentrations similar to those in smokers (Henningfield, 1995; Benowitz et al., 2001; Crowley-Weber et al., 2003). First of all, BLM-induced apoptosis was confirmed by various methods based on cell morphological alterations, DNA fragmentation, caspase cascade activation (results not shown) and, for the measurement of apoptosis, a cell counting method was chosen because it was fairly reproducible provided that cell smears being screened in the random hematological manner.

We have found that nicotinic acetylcholine receptors are likely to play a role in the increase of bleomycin-induced apoptosis by nicotine, which were blocked with nicotinic acetylcholine receptor antagonists such as mecamylamine, D-tubocurarine and α-bungarotoxin. The possible nature of the subunits involved in the nicotinic acetylcholine receptor point toward bungarotoxin sensitive receptors such as α7- or α9-nicotinic acetylcholine receptors. Since HL-60 cell lines are of hematological origin, the involvement of α7-subunits, but not α9, was suspected (Elgoyhen et al., 1994; Sato et al., 1999). Indeed, α7 mRNA was detected in HL-60 cell (Fig. 1). Stimulation of bleomycin-induced apoptosis by GTS-21, an agonist specific for α7-nicotinic acetylcholine receptor, and lack of effect with epibatidine and cytisine validate the involvement of α7-nicotinic acetylcholine receptor. Additionally, the effect observed with nicotine at concentration of 600 nM is compatible with the affinity of α7-nicotinic acetylcholine receptors for this drug. However, the wave of bleomycin-induced apoptosis produced by GTS-21 appears for a concentration lower (i.e. 60 nM) than that observed with nicotine. As affinity of homomeric nicotinic acetylcholine receptors for GTS-21 is thereabouts three to five times greater than for nicotine (Kem et al., 1997), it is not surprising to see the ''peak'' of induced apoptosis moving to lower concentrations with GTS-21.

The protective effect against bleomycin-induced apoptosis by the antagonists suggests the possible attendance of a hidden agonist such as acetylcholine which was described in T lymphocytes, but not in granulocytes (Bond et al., 1995), the line to which HL-60 cells belong. Inverse agonist activities were reported in myocardial adrenoreceptors (Varma et al., 1999; Fujii and Kawashima, 2001). They depended on allosteric changes of the receptors and could be considered as a hypothesis for our observation.

The increase of bleomycin-induced apoptosis by nicotine that we have observed contrasts with some observations reported in the literature (Wright et al., 1993; Li et al., 1999; Garrido et al., 2001; Hakki et al., 2001, 2002; Sugano et al., 2001). However, similar results were recently reported (Crowley-Weber et al., 2003) with the combination of nicotine and sodium deoxycholate, a cytotoxic salt bile. A certain similarity between this model and our biological system may be proposed, particularly for deoxycholate and bleomycin, both capable of producing the same cell stresses (Hamilton et al., 1995; Mailloux et al., 2001), and particularly of injuring the cell membrane.

In conclusion, the increase of the bleomycin-induced apoptosis by nicotine appears to be mediated through α7 nicotinic acetylcholine receptors. Such a model formed by HL-60/bleomycin/nicotine may be proposed as a tool for investigating α7 nicotinic acetylcholine receptor and their functionality implication in apoptosis.

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