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ORIGINAL RESEARCH

Nicotine Diminishes Alpha2-Adrenergic Receptor-Dependent Protection Against Oxidative Stress in H9c2 Cardiomyocytes

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Introduction: Nicotine is a major component of cigarette smoke with various detrimental cardiovascular effects, including increased oxidative stress in the heart. Agonism of α_2-adrenergic receptors (ARs), such as with dexmedetomidine, has been documented to exert cardioprotective effects against oxidative stress and related apoptosis and necroptosis. α_2-ARs are membrane-residing G protein-coupled receptors (GPCRs) that primarily activate Gi/o proteins. They are also subjected to GPCR-kinase (GRK)-2-dependent desensitization, which entails phosphorylation of the agonist-activated receptor by GRK2 to induce its decoupling from G proteins, thus terminating α_2AR-mediated G protein signaling.

Objective: In the present study, we sought to examine the effects of nicotine on α_2AR signaling and effects in H9c2 cardiomyocytes exposed to H_2O_2 to induce oxidative cellular damage.

Methods and Results: As expected, treatment of H9c2 cardiomyocytes with H_2O_2 significantly decreased cell viability and increased oxidative stress, as assessed by reactive oxygen species (ROS)-associated fluorescence levels (DCF assay) and superoxide dismutase activity. Both H_2O_2 effects were partly rescued by α_2AR activation with brimonidine in control cardiomyocytes but not in cells pretreated with nicotine for 24 hours, in which brimonidine was unable to reduce H_2O_2-induced cell death and oxidative stress. This was due to severe α_2AR desensitization, manifested as very low Gi protein activation by brimonidine, and accompanied by GRK2 upregulation in nicotine-treated cardiomyocytes. Finally, pharmacological inhibition of adenylyl cyclase (AC) blocked H_2O_2-dependent oxidative damage in nicotine-pretreated H9c2 cardiomyocytes, indicating that α_2AR activation protects against oxidative injury via its classic coupling to Gai-mediated AC inhibition.

Discussion/Conclusions: Nicotine can negate the cardioprotective effects of α_2AR agonists against oxidative injury, which may have important implications for patients treated with this class of drugs that are chronic tobacco smokers.

Keywords: α_2-adrenergic receptor, oxidative stress, cardiac myocyte, nicotine, GRK2, signal transduction, desensitization, α_2-adrenergic agonist, adenylyl cyclase, superoxide dismutase

Introduction

Cardiomyocyte death occurs during ischemia/reperfusion (I/R) injury via various processes, prominent among which are oxidative stress, intracellular Ca^{2+} overload, and inflammation.[1,2] Increased oxidative stress, in particular, is a chief cause of oxidative injury-induced apoptosis and inflammation.[3] Oxidative stress triggers numerous detrimental reactions leading to cell apoptosis and necrosis.[4,5] Tobacco smoking and, more specifically, its main toxic ingredient nicotine is known to induce cellular apoptosis and oxidative stress.[6,7]

α_2-adrenergic receptor (α_2AR) activation is widely used in perioperative patients for sedation and analgesia and has been explored as potential therapy for I/R injury in the myocardium, brain, and lungs.[8] α_2AR agonism might confer

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cardioprotection against oxidative injury, as recently demonstrated in the rat cardiomyoblast cell line H9c2, in which the α_2AR agonist dexmedetomidine significantly reduced hydrogen peroxide (H_2O_2)-induced oxidative stress and apoptosis.[8] α_2ARs consist of three different subtypes in humans (α_{2A}, α_{2B}, α_{2C}), all of which are G protein-coupled receptors (GPCRs) that couple mainly to Gi/o proteins to inhibit adenylyl cyclase (AC) and cyclic 3`, 5`-adenosine monophosphate (cAMP) synthesis in cells.[9,10] They are also essential for presynaptic autoinhibition of norepinephrine (NE) release from sympathetic neurons, as well as for autoinhibition of NE and epinephrine (Epi) secretion from the chromaffin cells of the adrenal medulla.[11,12] Importantly, they are subject, like most GPCRs, to GPCR-kinase (GRK)-dependent phosphorylation which leads to their desensitization, ie, decoupling from G proteins and signaling termination.[13–15] Indeed, one of the most abundant and ubiquitously expressed GRKs, GRK2, is upregulated in the adrenal medulla (as well as in the heart) during chronic heart failure (HF), resulting in severe α_2AR dysfunction and chronically increased catecholamine secretion from the adrenal gland in HF.[13–15]

In the present study, we sought to investigate the effects of nicotine on α_2AR-dependent cardio-protection against oxidative injury. To that end, we used H9c2 cells that endogenously express α_2ARs[8] treated with H_2O_2 to induce oxidative injury[16] after being exposed to nicotine. We found that nicotine can diminish the α_2AR-dependent protection against oxidative stress via GRK2 upregulation that dysregulates α_2AR signaling to AC inhibition.

Materials and Methods

Materials

All drugs and chemicals were from Sigma-Aldrich (St. Louis, MO, USA).

Cell Culture

The H9c2 rat cardiomyoblast cell line was purchased from American Type Culture Collection (Manassas, VA, USA) and cultured as previously described.[8,17] Briefly, H9c2 (embryonic rat heart-derived myoblast) cells were cultured in Dulbecco's modified Eagle medium (DMEM; GIBCO Laboratories, Grand Island, New York, USA) supplemented with 10% fetal bovine serum (FBS), streptomycin (100 mg/mL) and penicillin (100 units/mL) in a humidified incubator under the condition of 37 °C, 95% air and 5% CO_2.

Cell Viability Measurements

Cell viability was measured by using cell counting kit-8 (CCK-8, Sigma-Aldrich), as described.[8] First, optimal concentration of H_2O_2 was determined by constructing a dose–response curve for H_2O_2 (cells were exposed to 100 μM, 250 μM, 500 μM, 750 μM, and 1 mM H_2O_2 for 12 hrs). After confirming the optimal [H_2O_2] (500 μM), cells were pretreated with or without nicotine for 24 hours and then exposed to 10 μM brimonidine or DMSO. Thirty-minute later, 500 μM H_2O_2 was added for 10 hrs. For cell viability measurements, 6×10^3 cells were seeded in a 96-well plate and, upon completion of the experimental protocol, CCK-8 agent was added into each well at a ratio 1:10 (CCK-8: culture media) and incubated at 37 °C for 2 hrs. Absorbance reflecting viability of the cells was measured on a microplate reader at 450 nm. All measurements were performed in triplicate by a blinded examiner.

ROS DCF Assay

To determine reactive oxygen species (ROS) production, the 2′,7′-dichlorofluorescein diacetate (DCFDA) dye-based assay kit from Molecular Probes (Cat. #C13293; Eugene, OR, USA) was used, as previously described.[8,18] 0.5×10^6 cells were plated into 12-well plates. After agent treatment completion, cells were washed three times with PBS and incubated in serum-free medium with 2 μM DCFDA for 20 min. Fluorescence was observed under a fluorescence plate reader in which excitation and emission wavelengths were set at 495 and 520 nm, respectively. Fluorescence OD values obtained were normalized with protein determination and expressed as % of the values obtained in vehicle (1 μM DMSO)-treated cells.

SOD Activity Measurements

Activity of intracellular superoxide dismutase (SOD) was measured by Enzyme-linked immunosorbent assay (ELISA), essentially as described.[19] Briefly, 1×10^6 cells were seeded in a 6-well plate, and after treatment with agents, cells were harvested by centrifugation and resuspended in 1 × PBS after lysis via sonication, then centrifuged at 2200 rpm for 5 min at 4 °C. After protein determination (Bicinchoninic acid protein assay kit), the supernatant (100 µL) was used for SOD activity measurement using a kit (Cat. #ADI-900-157, Enzo Life Sciences, Farmingdale, NY, USA), and according to the manufacturer's instructions. Supernatant was mixed with SOD working solution (160 µL) at 4 °C for 30 min and the reaction mixture was centrifuged and transferred to 96-well plates, to measure the absorbance (reflecting SOD activity) at 450 nm.

Immunoblotting

H9c2 cell extracts were prepared, as described previously,[18] in a 20 mM Tris pH 7.4 buffer containing 137 mM NaCl, 1% Nonidet P-40, 20% glycerol, 10 mM PMSF, 1 mM Na_3VO_4, 10 mM NaF, 2.5 mg/mL aprotinin, and 2.5 mg/mL leupeptin. Protein concentration was determined, and equal amounts of protein per sample were used. Immunoblotting with antibodies for GRK2 (Cat. #sc-562) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Cat. #sc-25778; both from Santa Cruz Biotechnology, Santa Cruz, CA, USA) was performed and blots were visualized with enhanced chemiluminescence (ECL, Life Technologies, Grand Island, NY, USA) in the FluorChem E Digital Darkroom (Protein Simple, San Jose, CA, USA), again as previously described.[18] Densitometry was performed with the AlphaView software (Protein Simple) in the linear range of signal detection (on non-saturated bands).

Giα Activity Assay

Giα activation was determined on isolated plasma membranes from H9c2 cells using a HTRF (homogeneous time-resolved fluorescence)-based GTP Gi binding assay kit (Cat. #62GTPPET; Cisbio-PerkinElmer, Inc., Waltham, MA, USA), as previously described.[20] Briefly, this assay measures the level of Giα activation following agonist stimulation of a GPCR, thanks to the use of a europium-labeled GTP analog (Eu-GTP). During the activation process of the heterotrimeric Gi protein, GDP dissociates from the Giα subunit and gets replaced by the assay's fluorescent Eu-GTP analog that now binds to the Giα subunit, as the latter goes from its inactive to its active state. The fluorescent signal emitted by the Giα-bound Eu-GTP analog is detected via addition of an anti- Giα antibody, with the fluorescence intensity levels measured being directly proportional to the level of Giα activation.

Statistical Analyses

Data are presented as the mean± S.E.M. values. Statistical differences were assessed by one-way analysis of variance (ANOVA) (GraphPad Software, Inc., La Jolla, CA) followed by the Tukey's test for multiple comparisons. P values less than 0.05 were considered statistically significant.

Results

Nicotine Inhibits the Ability of Brimonidine to Reduce Oxidative Cell Death in Cardiomyocytes

After an initial dose–response titration (Supplementary Figure 1), we determined that 0.5 mM of H_2O_2 for 10 hours produced a robust and reliable cell death response in our cultured H9c2 cardiomyocytes. Therefore, we chose this concentration of H_2O_2 for all our subsequent experiments. As shown in Figure 1, the α_2AR full agonist brimonidine (UK 14304) conferred significant protection against hydrogen peroxide-induced cell death in normal control H9c2 cells. However, in nicotine-exposed cardiomyocytes, brimonidine was virtually unable to increase cell viability in the face of the H_2O_2 challenge (Figure 1). Of note, this inability of brimonidine was evident despite the fact that H_2O_2 appeared to be even more lethal/toxic to the nicotine-exposed cells compared to control H9c2 cells (Figure 1, compare cell viability between H_2O_2 and H_2O_2/nicotine-pretrated bars). Finally, this inability of brimonidine was not due to loss of α_2AR expression, as saturation radioligand binding experiments with [^{3}H]-rauwolscine[14] indicated similar B_{max} values for α_2ARs in control (17.1+2.3 fmol/mg protein) and H_2O_2-treated (16.3+3.2 fmol/mg protein) H9c2 cells.

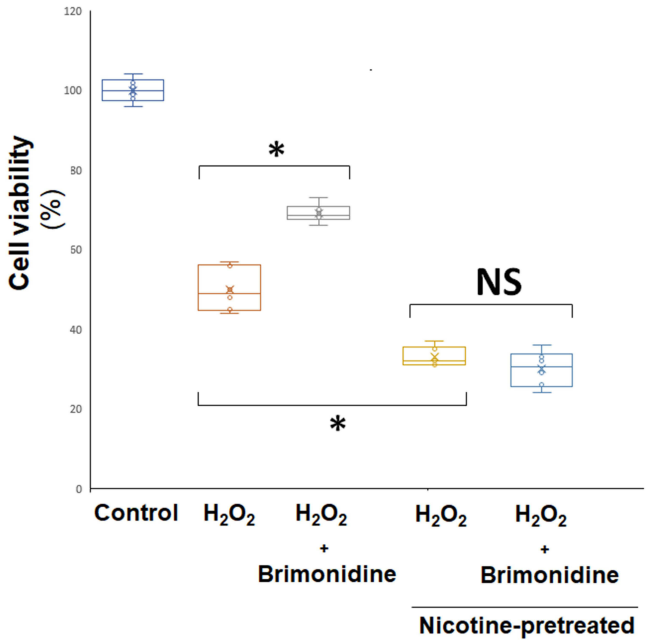

Figure 1 α_2AR activation and cell viability after nicotine exposure. H9c2 cardiomyocytes were treated with 1 μM nicotine or vehicle for 24 hours, followed by a 500 μM H_2O_2 application in the presence or absence of 10 μM brimonidine. 10 hours later, cell viability was determined. Results are expressed as % of control (no pretreatment and no agent application). *p<0.05; NS: Not significant at p=0.05; n=6 independent measurements per condition.

Nicotine Inhibits the Ability of Brimonidine to Reduce Oxidative Stress/Damage in Cardiomyocytes

Next, we examined the effect of nicotine exposure on H_2O_2-dependent oxidative stress and the ability of α_2ARs to protect against it. Brimonidine protected normal H9c2 cardiomyocytes against H_2O_2-dependent oxidative stress, as evidenced by the significant reduction in ROS levels (Figure 2A) and the preservation of SOD activity (Figure 2B) post-H_2O_2 challenge. However, in nicotine-exposed cardiomyocytes, it was again unable to reduce ROS levels (Figure 2A) or to increase SOD activity (Figure 2B) upon H_2O_2 application.

α_2ARs are Severely Desensitized Due to Elevated GRK2 Activity in Nicotine-Treated Cardiomyocytes

We next explored potential molecular mechanisms for the inability of α_2AR signaling to inhibit H_2O_2-induced oxidative stress in nicotine-exposed cardiomyocytes. Thus, we hypothesized that nicotine perhaps increases desensitization of α_2 ARs, thereby hindering their G protein signaling. Indeed, GRK2 was found upregulated in nicotine-exposed H9c2 cardiac myocytes (Figures 3A and B), and this was accompanied by severe desensitization of α_2ARs, as evidenced by the inability of brimonidine to activate Gi proteins in nicotine-pretreated cells (Figure 3C). Therefore, it appears that nicotine elevates GRK2-dependent α_2AR desensitization in H9c2 cardiomyocytes, which could underlie the failure of α_2AR agonism to protect against H_2O_2-induced apoptosis and oxidative stress.

Adenylyl Cyclase (AC) Inhibition Underlies α_2AR Anti-Oxidant Signaling in H9c2 Cardiomyocytes

AC activity, particularly that of AC5, one of the most abundant isoforms in cardiomyocytes, has been reported to promote oxidative stress and damage, while its inhibition or genetic deletion increases longevity and reduces aging by inhibiting oxidative stress and injury.[21–23] Given that α_2ARs inhibit AC via Gαi coupling and activation, we sought to test, in our last set of experiments, whether pharmacological inhibition of AC can rescue the severely desensitized α_2AR anti-oxidant signaling due to nicotine exposure. Indeed, AC inhibition with SQ 22536[24] was able to inhibit H_2O_2-induced

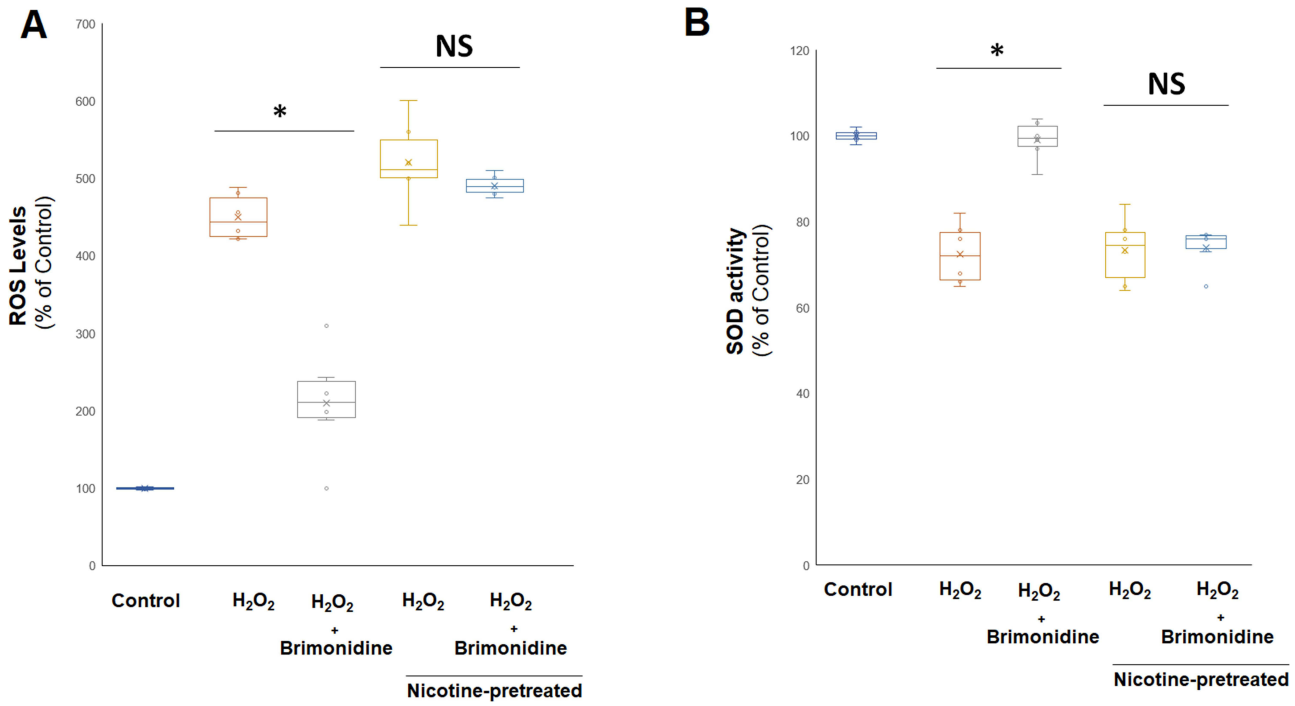

Figure 2 α_2AR activation and oxidative injury after nicotine exposure. H9c2 cardiomyocytes were treated with 1 μM nicotine or vehicle for 24 hours, followed by a 500 μM H_2O_2 application in the presence or absence of 10 μM brimonidine. 10 hours later, DCF fluorescence (ROS levels) and SOD activity were determined. (**A**) Quantification of ROS levels expressed as % of control (no pretreatment and no agent application). (**B**) SOD activity as % of control (no pretreatment and no agent application). *p<0.05; NS: Not significant at p=0.05; n=6 independent experiments per condition.

oxidative stress in nicotine-exposed H9c2 cardiomyocytes, either alone or in combination with brimonidine, as evidenced by markedly reduced ROS levels (Figure 4A) and significantly enhanced SOD activity (Figure 4B). This strongly suggests that α_2ARs protect against H_2O_2-induced oxidative damage via Gi protein-mediated AC inhibition.

Discussion

In the present study, we report that nicotine dysregulates sympatho-inhibitory α_2AR function via cardiac GRK2 upregulation (Figure 4C). This results in significant uncoupling of α_2ARs from Gαi-dependent AC inhibition, leading to unchecked oxidative stress development in cardiac myocytes (Figure 4C). Whether this signaling pathway is present in human cardiomyocytes in vivo is an open question, given that α_2ARs are the only AR type absent from human adult myocardium.[25] However, they are present and play major roles in cardiac presynaptic sympathetic nerve terminals regulating NE release.[26] Nevertheless, our present findings are in complete agreement with a recent study reporting the anti-oxidative properties of dexmedetomidine in H9c2 cardiomyocytes.[8] α_2ARs attenuate ROS accumulation stimulated by H_2O_2 exposure via mitochondrial and endoplasmic reticulum function improvements.[27] Consistent with this, we now show that brimonidine attenuates H_2O_2-induced ROS accumulation and preserves SOD activity, reduced by H_2O_2 exposure, in H9c2 cardiomyocytes.

However, nicotine pretreatment markedly reduced the efficacy of brimonidine towards these salutary effects against oxidative injury, indicating that nicotine can inhibit the ability of α_2ARs to afford anti-oxidant and anti-apoptotic cardio-protection. The mechanism for this appears to be GRK2 upregulation, which severely impairs α_2AR anti-oxidative signaling in H9c2 cardiomyocytes. Exactly how nicotine induces upregulation of GRK2 will be the focus of future studies. Most likely, however, it occurs at the transcriptional level, similarly to the adrenal βarrestin1 upregulation that nicotine also induces to elevate aldosterone production and cause hyperaldosteronism.[28,29] Our present study further expands this list with the addition of diminishing the anti-oxidative protection of adrenergic agonists in the heart courtesy of α_2AR dysfunction. Importantly, α_2ARs are downregulated in human HF,[10] contributing to the enhanced sympathetic nervous system activity that accompanies and aggravates human chronic HF. Since α_2ARs can also protect the heart

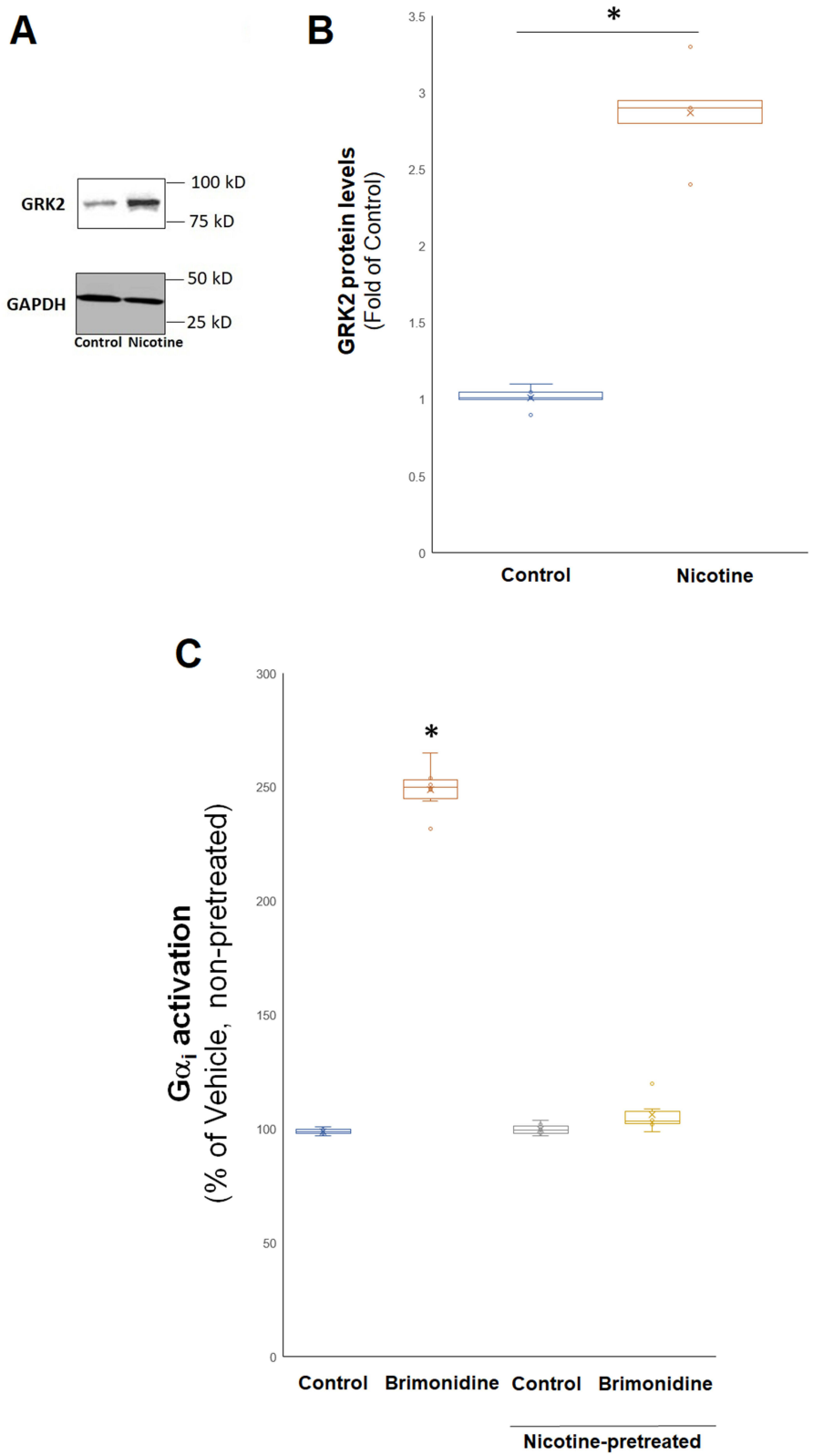

Figure 3 GRK2 levels and α_2AR signaling after nicotine exposure. H9c2 cardiomyocytes were treated with 1 μM nicotine or vehicle for 24 hours, followed by 10 μM brimonidine for 10 minutes. (**A** and **B**) Immunoblotting for GRK2 in protein extracts from these cells (GAPDH is also shown as loading control). A representative blot is shown in (**A**) and the densitometric quantification of five independent experiments in (**B**). *p<0.05; n=5. (**C**) $G\alpha_i$ activation in response to 10 μM brimonidine or vehicle (0.5% DMSO, Control) in plasma membranes isolated from these cells, expressed as % of vehicle in non-nicotine pretreated cells. *p<0.05; vs any other condition; n=6 independent experiments per condition.

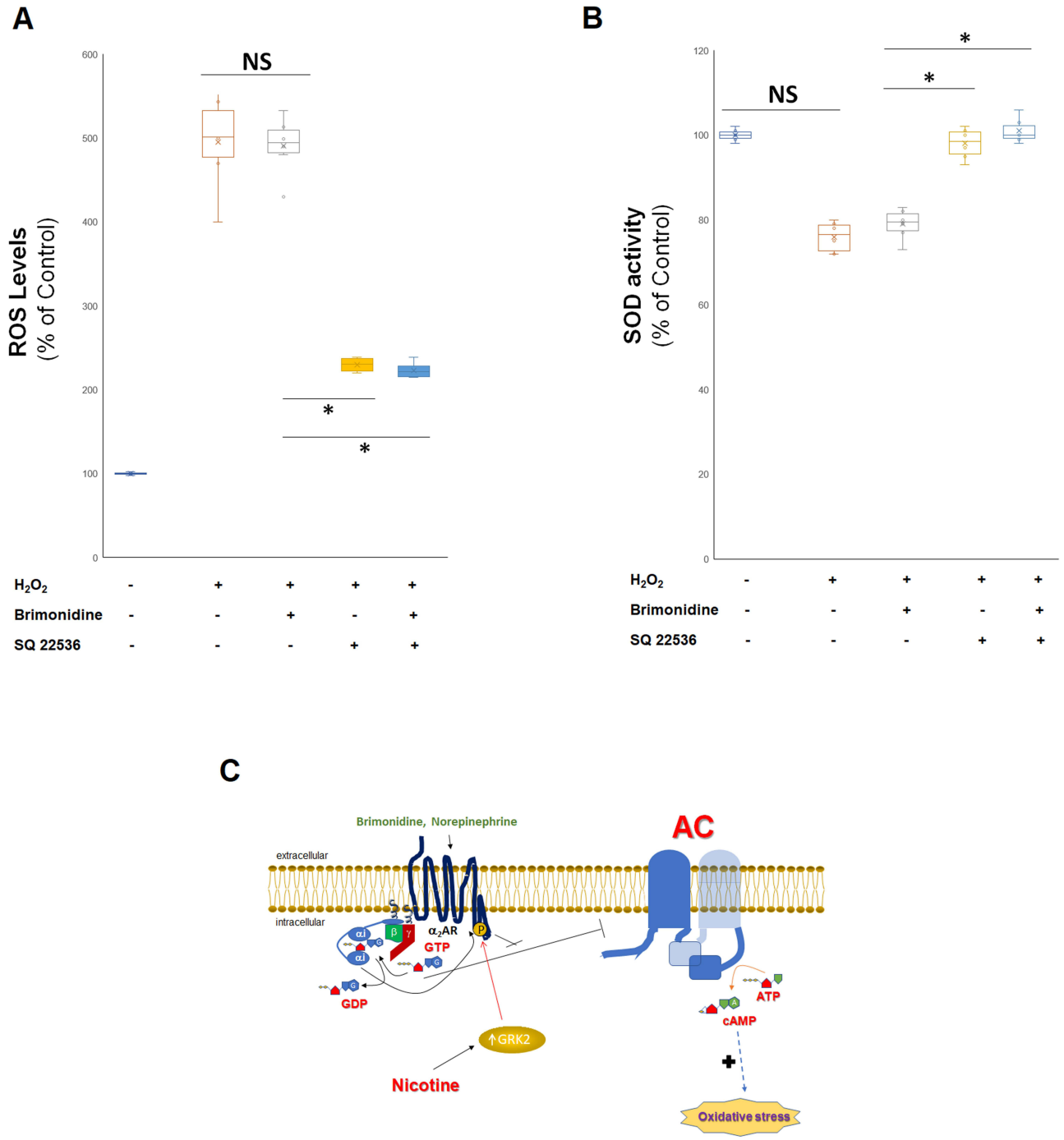

Figure 4 AC inhibition and α_2AR-regulated oxidative stress after nicotine exposure. H9c2 cardiomyocytes were treated with 10 μM nicotine for 24 hours, followed by a 500 μM H_2O_2 application in the presence or absence of 10 μM brimonidine or 0.2 mM SQ 22536 (AC inhibitor). 10 hours later, ROS levels (DCF fluorescence) and SOD activity were determined. (**A**) Quantification of ROS levels, expressed as % of control (vehicle, DMSO). (**B**) SOD activity as % of control (DMSO). *p<0.05; NS: Not significant at p=0.05; n=6 independent experiments per condition. (**C**) Proposed signaling pathway underlying α_2AR ant-oxidative protection in H9c2 cardiomyocytes and the role of nicotine. "+" denotes increase and the dotted blue arrow implies multiple steps leading to oxidative stress from cAMP. See main text for details.
Abbreviations: αI, Inhibitory G protein alpha subunit; GTP, Guanosine triphosphate; GDP, Guanosine diphosphate; P, Phosphorylation; ATP, Adenosine triphosphate; cAMP, Cyclic adenosine 3`, 5`-monophosphate.

against oxidative stress, their downregulation in human HF may represent one more reason for the increased oxidative stress present in human HF, especially if the patient is also a nicotine product user/smoker.

Regarding the cardioprotective signaling of α_2ARs against oxidative stress very little is known. Based on our present data (Figure 4) and those of others,[21–23,30–32] AC5 promotes ROS production and downregulates SOD, since AC5 knockout mice

live longer, age slower and their hearts seem protected from oxidative stress.[21–23,32] Interestingly, oxidative stress can also upregulate AC activity,[33] suggesting the existence of a positive feedback loop, at least in vascular smooth muscle.[33] On the other hand, cAMP is known to exert anti-oxidative effects in kidney cells[34,35] and cAMP-dependent protein kinase (protein kinase A, PKA) phosphorylates and inhibits nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) activator 1 (NOXA1), thereby suppressing ROS production by NOX1.[36] Therefore, the effect of AC on oxidative stress and ROS levels is most likely AC type- and cell/tissue type-dependent. In any case, the rescuing effect on anti-oxidative protection of the pan-AC type inhibitor SQ 22536 we observed in the present study strongly indicates that α_2ARs inhibit oxidative stress and increase SOD activity via Gi/o protein-mediated AC (specifically, AC5) inhibition in H9c2 cardiomyocytes. It is this inhibitory effect of α_2AR signaling that GRK2 opposes in order to confer the oxidative toxicity of nicotine in cardiac myocytes. These findings could also indicate that AC inhibitors (or GRK2 inhibitors) may be of therapeutic value in combating the deleterious effects of nicotine, the most important ingredient in tobacco, on cardiac oxidative stress. Alternatively, they can be used concomitantly with α_2AR agonists in chronic smokers to boost the efficacy of the latter drugs towards increasing cardiac survival after I/R injury, myocardial infarction, or septic cardiomyopathy.

Our present study has several limitations, the most important of which is that it was done exclusively in vitro and in non-fully differentiated cardiomyocytes (H9c2 cells are cardiac myoblasts that do not contract).[37] Another limitation is that we did not examine which of the three α_2AR subtypes is expressed in H9c2 cardiomyocytes and mediates the anti-oxidative effects of brimonidine. This is potentially significant, since the three α_2AR subtypes are pharmacologically distinct and may display differences in their susceptibility to GRK-dependent desensitization.[12,13]

In conclusion, the present study reports, for the first time to our knowledge, that nicotine can perturb α_2AR signaling towards cardio-protection against oxidative stress through GRK2 upregulation and that this can be rescued by AC pharmacological inhibition. Pending confirmation in vivo, these findings could have significant clinical ramifications for the pharmacotherapy of patients on α_2AR agonists, such as those in ICU or patients treated for addiction, who are also nicotine/tobacco users/smokers.

Data Sharing Statement

All source data files are available upon request to the correspondence author.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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