Atrial Natriuretic Peptide Attenuates Hypoxia Induced Chemoresistance in Prostate Cancer Cells

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Purpose: Low tumor oxygenation (hypoxia) correlates with resistance to chemotherapeutic agents. We recently reported that in vitro hypoxia induced resistance to various anti-cancer drugs can be attenuated by nitric oxide mimetic agents. Natriuretic peptides are molecules that mediate their cellular effects by activating a signaling pathway similar to that activated by nitric oxide. In the current study we determined whether atrial natriuretic peptide is able to inhibit hypoxia induced chemoresistance in prostate carcinoma cells.

Materials and Methods: Reverse transcriptase-polymerase chain reaction and atrial natriuretic peptide binding studies were used to determine the presence and function of natriuretic peptide receptors on a panel of human cell lines as well as in tissue samples. Drug sensitivity assays of cell lines exposed to hypoxic or standard conditions were performed in the presence of various concentrations of atrial natriuretic peptide.

Results: These studies revealed the presence of the 3 known natriuretic peptide receptors A, B and C in PC-3 and DU-145 human prostate carcinoma cells (American Type Culture Collection, Manassas, Virginia) as well as in tissue samples of human prostate cancer. Atrial natriuretic peptide binding to these cells was unaffected by culture in 0.5% vs 20% O_2. Clonogenic assays revealed that incubation of these cells in 0.5% O_2 for 24 hours resulted in a subsequent 4 to 10-fold increase in their survival following 1-hour exposure to doxorubicin (Sigma®) (12.5 μM) ($p < 0.001$). While small concentrations of atrial natriuretic peptide (10^{-7} to 10^{-13} M) did not affect sensitivity to doxorubicin in cells incubated in 20% O_2, similar concentrations of atrial natriuretic peptide inhibited the survival of these cells incubated in 0.5% O_2 by up to 50% ($p < 0.006$). Using the cyclic guanosine monophosphate dependent protein kinase G inhibitor KT5823 (15 μM) the chemosensitizing effect of atrial natriuretic peptide was abrogated.

Conclusions: These results indicate the potential use of natriuretic peptides as adjuvants to chemotherapy for prostate cancer.

Key Words: prostate, prostatic neoplasms, drug resistance, atrial natriuretic factor, anoxia

Tissue hypoxia characterizes many solid tumors and it is proposed to be an independent prognostic indicator of poor clinical outcome in patients with various cancers, including prostate cancer.[1,2] Tumor hypoxia also correlates with increased tumor invasion and metastasis[3–5] as well as with resistance to certain chemotherapeutic agents.[6–8] Hypoxia induced chemoresistance is likely multifactorial and it could be attributable in part to a lack of O_2 available for drug action, increased genetic instability, antiproliferative hypoxia effects[9] and an increase in the activity of the multidrug resistance transporter P-glycoprotein.[7]

Our previous studies revealed that sensitivity to chemotherapeutic agents can be reestablished in hypoxic tumor cells by administering small concentrations of NO mimetic agents. Those studies also showed that pharmacological inhibition of endogenous NO production in well oxygenated cells leads to a drug resistance phenotype in a manner similar to exposure to hypoxia.[8] Thus, we postulated that, since O_2 is required for the formation of NO, a decrease in endogenous NO dependent signaling in a hypoxic environment underlies hypoxia induced chemoresistance.

An important mechanism by which NO exerts its biological effects involves the binding to and activation of intracellular soluble GC. This leads to increased intracellular cGMP levels and downstream activation of cGMP dependent PKG.[10] In recent studies we noted that exposure of human breast carcinoma cells to hypoxia decreased intracellular cGMP levels.[4] This decrease in cGMP levels was causally linked to increased invasion through extracellular matrix and increased expression of uPAR, a molecule required for tumor invasion and metastasis,[11] since administration of a nonhydrolysable analogue of cGMP (8-bromo-cGMP) or NO mimetics inhibited hypoxia induced invasiveness and uPAR expression.[4] Moreover, in a manner similar to exposure to hypoxia the inhibition of soluble GC or PKG activity increased uPAR expression.[4]

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There are several plasma membrane enzymes that synthesize the second messenger cGMP, although ligands have only been identified for 3 of them, of which 2 are natriuretic peptides.[12] Natriuretic peptides, including ANP, are a family of polypeptides that cause diuresis/natriuresis and vasodilation, among other important physiological roles.[13] Of the 3 NPRs 2 (A and B-type or GC-A and GC-B) act via membrane bound particulate GC to activate a cGMP dependent signaling cascade.[13–15] Because of the commonality between the NO and ANP pathways, we hypothesized that ANP attenuates hypoxia induced chemoresistance and, therefore, it could be used as adjuvant therapy in chemotherapeutic regimens in patients with advanced prostate cancer.

MATERIALS AND METHODS

Cells

Human PC-3 and DU-145 prostatic adenocarcinoma cells were used. PC-3 cells were maintained in monolayer culture in the Kaighn modification of Hamm's F12 medium supplemented with 10% fetal bovine serum. DU-145 cells were maintained in RPMI-1640 medium supplemented with 5% fetal bovine serum (Gibco Invitrogen™). MDA-MB-231 cells were used to compare ANP binding and the ANP effect on cell survival after exposure to chemotherapy.

Culture Conditions

For incubation in standard culture conditions (20% O_2) cells plated in 6-well culture plates (70% to 80% confluent) were placed in a Sanyo® CO_2 incubator (5% CO_2 in air at 37C). Hypoxic conditions were established, as described previously.[4,8] Briefly, cells in tissue culture plates were placed in airtight chambers (BellCo Biotechnology, Vineland, New Jersey) that were flushed with a gas mixture of 5% CO_2/95% N_2. Oxygen concentrations in these chambers were maintained for 24 hours at 0.5% using Pro-Ox Model 110 O_2 regulators (Biospherix, Redfield, New York). We previously determined that the O_2 concentration at the bottom of the tissue culture plate equilibrates with atmospheric O_2 levels within 2 hours of incubation under hypoxia.[16] Randomly selected culture plates were then incubated with various concentrations of ANP (10^{-7} to 10^{-15} M) administered once at the beginning of hypoxic exposure.

Human Prostate Tissues

Human prostate tissues were obtained after radical prostatectomy. Areas of prostatic adenocarcinoma from 3 patients, benign prostatic tissue (glandular atrophy) or PIN were identified in dissected histological sections of the tissues.

RNA Isolation

Total RNA was extracted from frozen tissues or cell pellets by a modification of the acid guanidinium-phenol-chloroform method using TriReagent (Sigma) according to manufacturer instructions. Precipitated RNA was resuspended in RNase-free (dihexadecylphosphatidylcholine treated) water and stored at −20C until use for cDNA synthesis.

RT-PCR

First strand cDNA was transcribed from 1 μg total RNA using an Omniscript™ RT Kit according to manufacturer instructions. Briefly, a master mix was freshly prepared by adding reaction components, including 10 × RT buffer, deoxynucleoside triphosphate mix, Oligo-dT18 primer (Cortec DNA Services, Kingston, Ontario, Canada), RNase inhibitor (MBI Fermentas, Burlington, Ontario, Canada), Omniscript RT and RNase-free water. RNA from each sample was added to this mix and allowed to incubate for 60 minutes at 37C. Resultant cDNA was stored at 4C until use. Each PCR was performed in 30 μl containing certain reagents, including 0.5 μl cDNA product, 1 × Taq Polymerase Buffer, 2.5 mM $MgCl_2$, 1 × Q Solution, 1 ρmol sense and antisense primers, 2.5 U Taq Polymerase (Qiagen™) and 10 mM deoxynucleoside triphosphates (MBI Fermentas). DNA sequences for each gene were obtained from the GenBank database (http://www.ncbi.nlm.nih.gov). Primers for PCR amplification were designed with Primer Designer 2.01 (Scientific and Educational Software, Durham, North Carolina).

The sequences of each gene specific primer were human NPR-A sense primer 5′-CGGTGTCAAGGACGAGT-3′ and antisense primer 5′-ACAAGCCAGCTTCCAGG-3′, human NPR-B sense primer 5′-CTGGCTGCTTCTATGAT-3′ and antisense primer 5′-TGAGCGAGCCGTAACT-3′, and human NPR-C sense primer 5′-GCTGGCTGCTTCTATGAT-3′ and antisense primer 5′-ATGAGCGAGCCGTAACT-3′. PCR reactions were done on an EP gradient Thermocycler (Eppendorf, Westbury, New York). PCR amplicons were loaded on 1% agarose TBE gel (Bioshop, Mississauga, Ontario, Canada) and stained with ethidium bromide.

Receptor Binding Studies

To help determine the functionality of the natriuretic peptide surface receptors on the cancer cell lines binding assays were performed under 20% and 0.5% O_2 in serum-free RPMI (Gibco) with 0.1% bovine serum antigen (Sigma). Cells were grown to 90% confluence and competitive binding assays were initiated by adding 100,000 cpm ^{125}I-ANP per plate. To determine nonspecific binding of receptors binding assays were done in the presence of 1 μM human ANP (American Peptide, Sunnyvale, California). In another set of plates the specific ligand C-ANP$^{4-22}$ (American Peptide), which selectively blocks the binding of NPR-C, was added at a concentration of 1 μM to determine the abundance of GC coupled receptors (NPR-A and B). Finally, to determine total binding 0.1 M acetic acid was added to a third set of plates. Cultures were incubated with ANP at 4C for 60 minutes. Wells were washed 3 times with PBS and cells were harvested with 0.075% trypsin-ethylenediaminetetraacetic acid in PBS. Detached cells and supernatants were counted with a Beckman™ Gamma 5500B γ counter.

Exposure to Doxorubicin and Clonogenic (Colony Formation) Assay

Following pre-incubation of cells in 20% or 0.5% O_2 in the presence or absence of ANP cultures were incubated with doxorubicin (0 to 25 μM) for 1 hour at 20% O_2 in a standard CO_2 incubator. Cultures were washed with drug-free PBS. Cells were harvested with 0.075% trypsin-ethylenediaminetetraacetic acid in PBS and counted using a hemocytometer. Survival of cancer cells following doxorubicin exposure was determined by a clonogenic assay, as previously described.[8] After 7 to 14 days colonies were fixed with acetic acid-methanol (1:4) and stained with a dilute crystal violet solution (0.33% weight per volume) before being counted. To

determine the mechanism of any effect of ANP on hypoxia induced chemoresistance the cGMP dependent PKG inhibitor KT5823 was used in similar colony formation assays.

Calculations and Statistical Analyses

Plating efficiency was calculated from the number of surviving colonies expressed as a proportion of the total number of cells seeded. Surviving fractions were determined by dividing the plating efficiency of drug treated groups with the plating efficiency of their respective control groups. All data are presented as the mean ± SD. Statistical analyses were performed using StatView® statistical software. Statistical significance was determined by 1-way ANOVA followed by Fisher's post hoc analysis. All statistical tests were 2-sided and differences were considered statistically significant at $p < 0.05$.

RESULTS

Presence of NPRs on Cell Lines and Human Prostate Tissue

RT-PCR confirmed the presence of all 3 NPRs in the prostate cancer cell lines tested as well as in the dissected prostate tissue obtained after radical prostatectomy, including benign tissue and cancer (fig. 1). Competitive binding studies demonstrated that the C-receptor represented approximately 30% of total ANP binding in PC-3 cells (data not shown) and DU-145 cells (fig. 2, *A*). Culturing DU-145 cells in 0.5% O_2 did not affect ANP binding (fig. 2, *C*). As a comparison, NPR status and binding studies were performed in the MDA-MB-231 human breast cancer cell line. This cell line also expressed all 3 NPR transcripts (data not shown). The studies also demonstrated a similar proportion of binding of A and B type receptors (those with intrinsic GC activity) in MDA-MB-231 cells. This binding was not affected by culturing the cells in 0.5% O_2 (fig. 2, *D*).

Exposure to Hypoxia and Drug Resistance

Clonogenic assays revealed that, compared with incubation in 20% O_2, incubation of DU-145 and MDA-MB-231 cells in 0.5% O_2 for 24 hours resulted in a corresponding 4 and 10-fold increase in survival following 1-hour exposure to doxorubicin ($p < 0.001$, figs. 3 and 4). The highest level of hypoxia induced chemoresistance was observed with 12.5 μM doxorubicin and subsequently this concentration was used in most ANP studies. The plating efficiency of DU-145 and MDA-MB-231 cells was approximately 70% to 90%, although cells incubated in hypoxia had an expected decrease in plating efficiency (average 50% to 70%).

Effect of ANP on the Induction of Hypoxia Associated Drug Resistance

The relative survival of DU-145 prostate cancer cells following exposure to doxorubicin (12.5 μM) was unaffected by a single administration of ANP when cells were incubated in 20% O_2 (fig. 3). However, similar incubation with various concentrations of ANP at the onset of the 24-hour incubation in 0.5% O_2 significantly decreased the magnitude of hypoxia induced drug resistance in DU-145 cells ($p < 0.0001$, fig. 3). Statistically significantly decreased survival after ANP exposure was also observed in MDA-MB-231 cells (fig. 4). A doxorubicin dose-response study (0 to 25 μM) was also performed with an ANP concentration of 10^{-11} M in hypoxic conditions. Relative cell survival went from 100% without incubation in doxorubicin to 0.2% when incubated with the maximum dose of 25 μM doxorubicin. A decrease in relative cell survival was noted at each concentration (data not

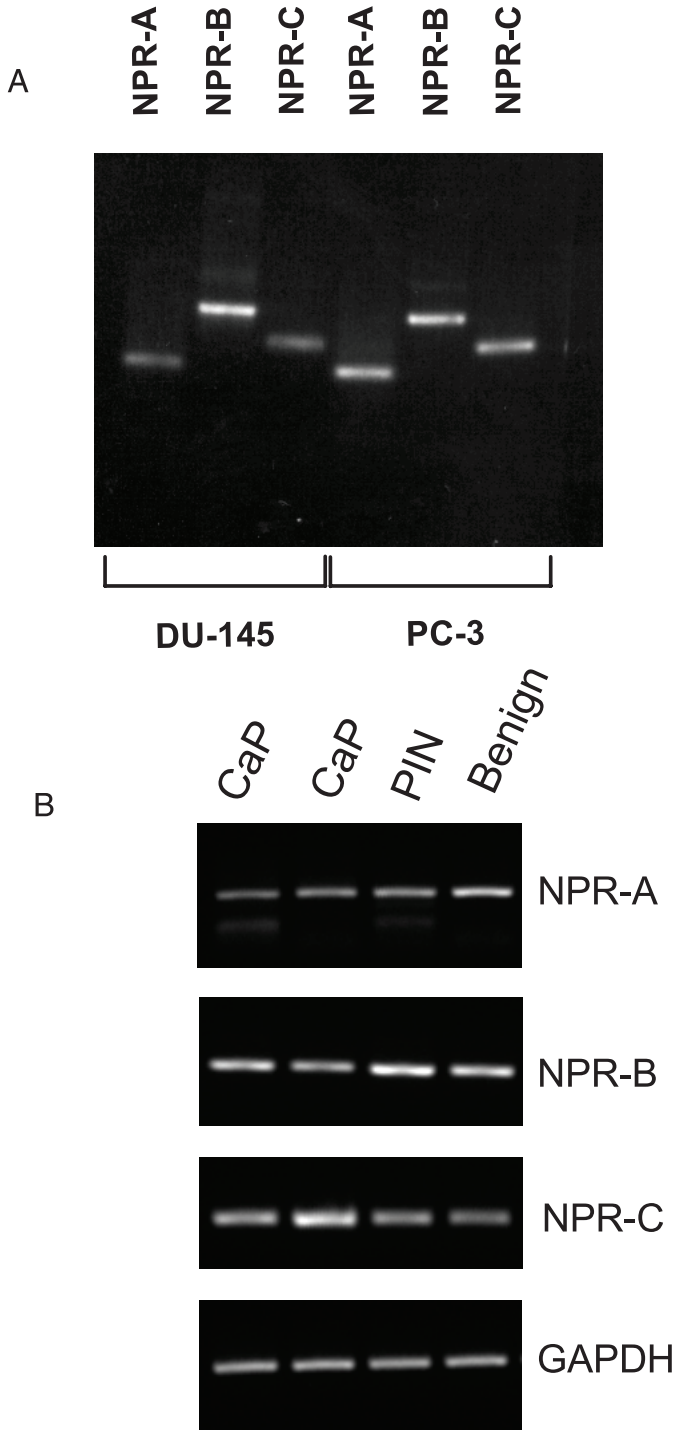

FIG. 1. NPR expression in cultured prostate cancer cell lines and human prostate tissue. NPR-A, B and C transcript RNA in DU-145 and PC-3 prostate cancer cell lines were measured by RT-PCR (*A*). Note NPR mRNA expression in prostate tissue dissected from radical prostatectomy specimens (*B*). PIN indicates dissected high grade PIN area in prostate of patient with prostate cancer. *Benign*, mRNA from prostatectomy specimen tissue showing only glandular atrophy. *CaP*, NPR transcript from areas of Gleason 5/10 prostatic adenocarcinoma.

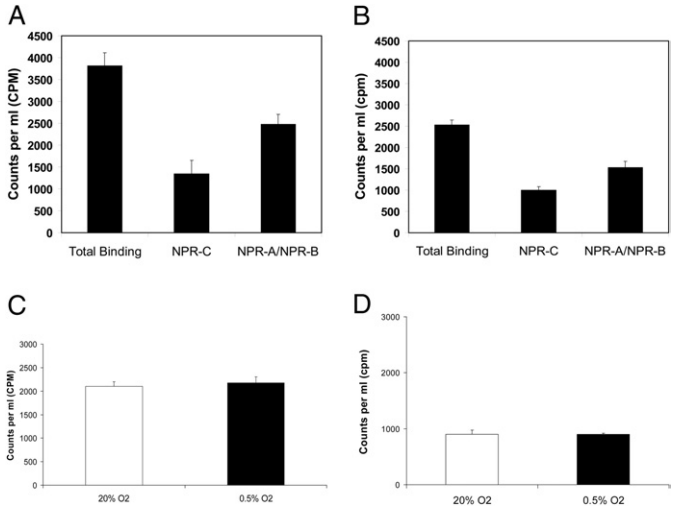

FIG. 2. Relative NPR levels and effect of O_2 concentration in binding studies. Relative abundance of NPR-A and B (with GC activity) compared to NPR-C receptors in DU-145 (*A*) and MDA-MB-231 (*B*) cells in binding studies is shown by ^{125}I labeled ANP in cpm. Relative abundance of combined NPR-A and B receptors expressed on DU-145 (*C*) and MDA-MB-231 (*D*) cells cultured in 20% or 0.5% oxygen is represented by ^{125}I labeled ANP total cpm. Data points indicate mean ± SD cpm, representing typical response in assay done at least in quadruplicate for all experiments.

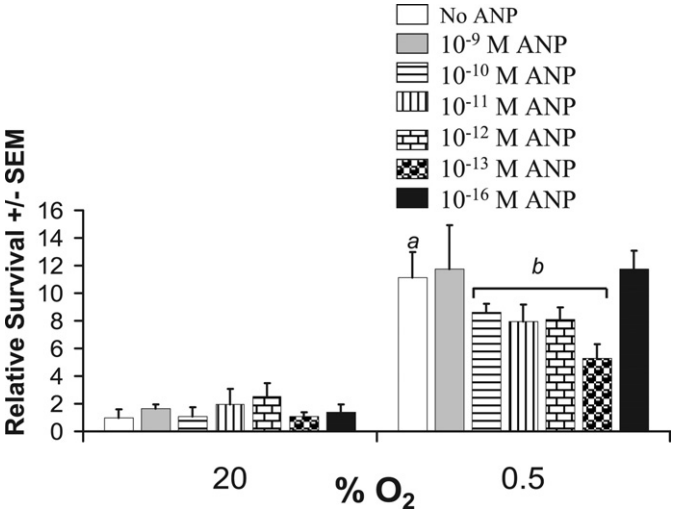

FIG. 4. Effect of ANP on hypoxia induced resistance to doxorubicin (12.5 μM) in MDA-MB-231 breast carcinoma cells treated with doxorubicin for 1 hour following 24-hour incubation in 0.5% or 20% O_2. Compared with incubation in 20% O_2 incubation in hypoxia alone resulted in significant increase in survival (*a*, $p < 0.001$) Significant attenuation of hypoxia induced chemoresistance was noted with 10^{-10} to 10^{-13} M ANP (*b*, $p < 0.01$). Results showing effect of hypoxia on survival in absence of ANP treatment are representative of 3 independent experiments performed in triplicate wells. Results showing effect of ANP treatment are representative of 2 experiments done in triplicate wells. Data points represent mean relative surviving fraction ± SEM.

shown). The plating efficiency of cells did not differ significantly with and without ANP administration. Treating DU145 cells with the PKG inhibitor KT5823 (15 μM) in the presence of ANP resulted in significant abrogation of the ANP chemosensitizing effect (fig. 5).

DISCUSSION

The main finding of the current study is that low concentrations of ANP significantly attenuate hypoxia induced resistance to doxorubicin in cancer cells expressing functional NPR. Specifically results reveal that exposure to hypoxia resulted in resistance to doxorubicin in cancer cells and that this resistance was decreased by nanomolar concentrations of ANP. Furthermore, inhibiting PKG prevented the actions of ANP. Findings also provide evidence that all 3 ANPRs (NPR-A, B and C) were expressed in human prostate and breast cancer cell lines as well as in samples of human prostate cancer tissue. Furthermore, we confirmed significant binding of ANP to NPR-A and B (those with guanylyl

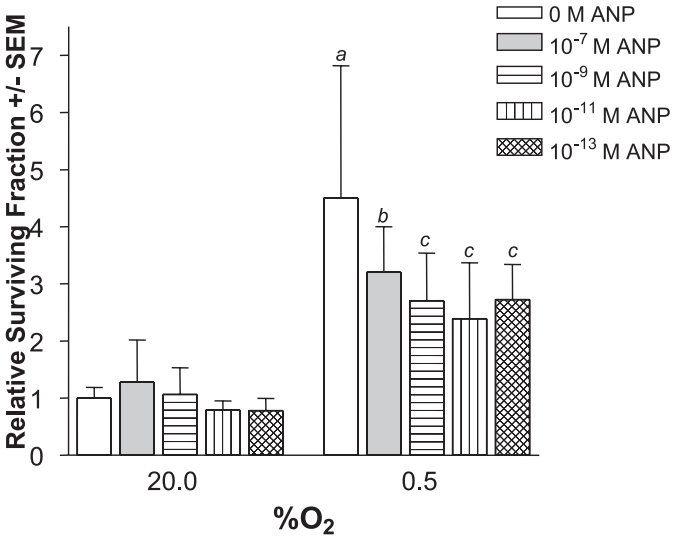

FIG. 3. Effect of ANP on hypoxia induced resistance to doxorubicin (12.5 μM) in DU-145 prostate cancer cells, as determined by colony forming ability. Compared with incubation in 20% O_2 without ANP incubation in hypoxia resulted in significant increase in survival (*a*, $p < 0.0001$). Treatment of cells with various ANP concentrations significantly attenuated acquisition of hypoxia induced resistance to doxorubicin following single 24-hour incubation in hypoxia compared with cells incubated in 0.5% O_2 alone (*b* and *c*, $p < 0.001$ and < 0.0001, respectively). Data points represent mean relative surviving fraction ± SEM of 3 independent experiments done in triplicate.

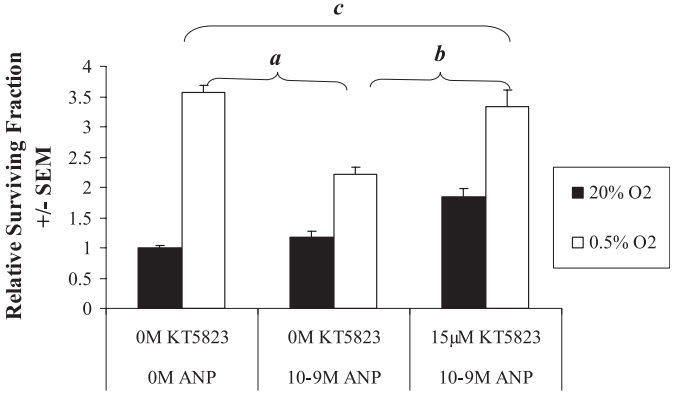

FIG. 5. Effect of cGMP dependent PKG inhibition on ANP mediated chemosensitization of hypoxic DU-145 cells treated with doxorubicin for 1 hour following 24-hour incubation in 0.5% or 20% O_2. Comparative groups were treated with or without 10^{-9} M ANP and 15 μM KT5823. Significant attenuation of hypoxia induced chemoresistance was noted with 10^{-9} M ANP (*a*, $p < 0.001$). PKG inhibitor significantly inhibited this response of ANP (*b* and *c*, $p < 0.001$ and 0.425, respectively). Results represent pooled findings in 3 separate experiments. Data points represent mean relative surviving fraction ± SEM.

cyclase activity) in MDA-MB-231 and DU-145 cancer cell lines.

Concentrations of ANP of 10^{-7} to 10^{-13} M effectively attenuated hypoxia induced resistance to doxorubicin in both cell lines. In dose-response studies 10^{-11} M ANP was effective in decreasing the effective concentration of doxorubicin necessary for cell killing. In MDA-MB-231 cells ANP had a significantly greater chemosensitizing effect at 10^{-13} M than at higher concentrations. A possible explanation for this U-shaped dose effect is that through increased cGMP production higher ANP concentrations may lead to protein kinase A activation.[17] There is evidence that the cAMP/protein kinase A cascade can inhibit cGMP mediated responses, such as neuronal migration.[18] In support of this concept we observed a similar inverse dose effect using the NO mimetics nitroglycerin and DETA-NO ((z)-1-[2-(2-aminoethyl)-*N*-(2-ammonioethyl)amino] diazen-1-ium-1,2 diolate) or the nonhydrolysable analogue of cGMP 8-bromo-cGMP (unpublished data). The PKG inhibitor KT5823 was able to prevent the chemosensitizing effects of ANP. This indicates that increased intracellular cGMP levels result in PKG activation and the attenuation of hypoxia associated chemoresistance.

In our study pre-incubation of cells in 0.5% O_2 resulted in a several fold increase in clonogenic ability following 1-hour exposure to doxorubicin. We previously observed a similar increase in hypoxia induced drug resistance in human PC-3 and mouse TRAMP-C2 cancer cells exposed to doxorubicin.[6] In the current study we incubated cells in 0.5% O_2 (less than 5 mm Hg) based on the rationale that these levels are characteristic of tissues with compromised blood flow, such as solid tumors, including prostatic carcinomas.[2] While our controls consisted of cells incubated in a relatively hyperoxic environment (20% O_2 or 152 mm Hg), our previous studies demonstrated that the sensitivity to doxorubicin of MDA-MB-231 cells incubated in 20% O_2 is identical to that of cells incubated in more physiological 5% O_2 concentrations (38 mm Hg).[8]

In this study the chemosensitizing effect of ANP was specific to cells incubated in 0.5% O_2 and cells incubated in 20% O_2 remained unaffected. Similarly our previous studies demonstrated that low concentrations of nitroglycerin only chemosensitized cells pre-exposed to hypoxia and not cells maintained in 20% O_2.[6,8] This selectivity of nitroglycerin and ANP to hypoxic cells is clinically important because any therapeutic approach designed to chemosensitize tumors should target hypoxic, drug resistant cell populations.

Interestingly all NPRs were found to be expressed in human prostate tissue and in the commonly used DU-145 and PC-3 cell lines. ANP specific receptors are strategically expressed in many normal tissues to aid in its primary function as a regulator of fluid balance and blood pressure.[13] The presence of NPRs was also reported in human adrenal tumors and hepatoblastomas.[19,20] In this study the binding of ANP to NPR-A/B in the 2 prostate cancer cell lines was not affected by O_2 levels. Activation of NPR-A and B leads to increased intracellular cGMP due to their intrinsic GC activity.[12–15] Therefore, they are most interesting in terms of exploring cGMP mediated adaptive responses to hypoxia. Previous studies showed that ANP increases GC activity by 150%.[14] ANP has the greatest binding affinity to NPR-A, whereas CNP has the greatest binding affinity for NPR-B.[13] The ANP prohormone contains 3 other peptide hormones, including long-acting natriuretic peptide, vessel dilator and kaliuretic peptide, which have significant variation in clinical effects for managing congestive heart failure.[21] Further study of the effect of these other peptide hormones on chemosensitivity appears warranted. Furthermore, Vesely et al previously observed that ANP and these other peptide hormones can inhibit the growth of several cancer cell lines, including those of the breast and prostate, and it is associated with decreased activation of ERK1 and ERK2.[22] Further study is needed to examine the potential role of ERK/mitogen activated protein kinase signaling in hypoxia induced chemoresistance.

CONCLUSIONS

The current study provides evidence for a role of ANP in the attenuation of chemoresistance in hypoxic cancer cells. Further study is indicated to determine if natriuretic peptides may be a useful approach to chemosensitize hormone refractory prostate cancer.

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Abbreviations and Acronyms

ANP	=	atrial natriuretic peptide
ERK	=	extracellular receptor kinase
cGMP	=	cyclic guanosine monophosphate
GC	=	guanylyl cyclase
GTN	=	glyceryl trinitrate
NO	=	nitric oxide
NPR	=	natriuretic peptide receptor
PBS	=	phosphate buffered saline
PCR	=	polymerase chain reaction
PKG	=	protein kinase G
RNase	=	ribonuclease
RT	=	reverse transcriptase
uPAR	=	urokinase plasminogen activator receptor

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