Doxorubicin Selectively Inhibits Brain Versus Atrial Natriuretic Peptide Gene Expression in Cultured Neonatal Rat Myocytes

Songcang Chen, Miklos Garami, David G. Gardner

Abstract — Doxorubicin is an antineoplastic agent with significant cardiotoxicity. We examined the effects of this agent on the expression of the natriuretic peptide (NP) genes in cultured neonatal rat atrial myocytes. Doxorubicin suppressed NP secretion, steady-state NP mRNA levels, and NP gene promoter activity. In each instance, brain NP (BNP) proved to be more sensitive than atrial NP (ANP) to the inhibitory effects of the drug. ICRF-187 and probucol reversed the inhibition by doxorubicin of ANP mRNA accumulation and ANP gene promoter activity while exerting no effect on BNP mRNA levels or promoter activity. This represents the first identification of the NP genes as targets of doxorubicin toxicity in the myocardial cell. This inhibition operates predominantly at a transcriptional locus and has more potent effects on BNP versus ANP secretion/gene expression. Measurement of BNP secretion/gene expression may provide a sensitive marker of early doxorubicin cardiotoxicity. (Hypertension. 1999;34:1223-1231.)

Key Words: doxorubicin ■ natriuretic peptides ■ hypertrophy ■ cardiomyopathies

Doxorubicin is an anthracycline antibiotic that is used clinically in the management of a variety of leukemias and solid tumors. Despite its potent antineoplastic activity, its use is limited by the dose-related cardiotoxicity associated with the drug. This toxicity is characterized by progressive myocyte damage that can lead to dilated cardiomyopathy and refractory congestive heart failure. The mechanisms that underlie the cardiotoxicity are only partially understood. Possible mechanisms include direct or indirect release of endogenous toxins (eg, histamine), alterations in intracellular calcium homeostasis, generation of free radicals that damage cellular membranes, and intercalation of drug in the nuclear and mitochondrial genome, resulting in diminished RNA and protein synthesis. Of these mechanisms, the free radical hypothesis has received the most support. It is thought that doxorubicin, through its semiquinone metabolite, generates superoxide anion and superhydroxide free radicals by using intracellular iron as a cofactor. Because the heart is relatively deficient in those enzymes responsible for clearing free radicals (ie, superoxide dismutase, catalase, and glutathione peroxidase), the administration of doxorubicin may lead to significant lipid peroxidation and destruction of mitochondrial membranes.

Dexrazoxane (ICRF-187), a chelator of intracellular iron, and probucol, an antioxidant with hypolipidemic properties, have each been shown to offer protection against the cardiomyopathic properties of doxorubicin in animal and selected human studies. However, neither dexrazoxane nor probucol offers complete protection from the cardiomyopathic effects of doxorubicin. Thus, a more detailed understanding of the mechanisms underlying this toxicity may lead to better methods for early detection of cardiac dysfunction and the design of effective therapeutic strategies to limit its progression.

Atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) are hormones that are produced and secreted predominantly from the myocytes of the heart. ANP is preferentially expressed in the atrium but is found at low levels in adult ventricular myocytes. BNP is more uniformly distributed between atrial and ventricular myocardium. The expression of both ANP and BNP genes is activated early in the process of myocyte hypertrophy. The high degree of fidelity with which this activation occurs has led to their use as markers of the hypertrophic process in a variety of in vitro, whole-animal, and clinical models.

Bauch et al reported elevations of plasma ANP in pediatric patients 3 to 5 weeks after doxorubicin treatment. A more detailed animal study from Bernardini et al found an acute reduction in plasma ANP levels after treatment of intact female Wistar rats with a single dose of doxorubicin. Thus, the nature of the effect of doxorubicin on ANP gene expression and secretion is poorly defined, and effects on BNP expression have yet to be reported. We have used an in vitro model of cultured neonatal rat atrial myocytes to study the effect of doxorubicin on the expression of the natriuretic peptide (NP) genes. Our findings suggest that doxorubicin...
Effect of Dox on Atrial Cell Viability

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<td>48</td>
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Values are mean ± SD from 3 different experiments. Atrial myocytes were cultured in DMEM/SS with 5 mg/L actinomycin D (Act D, Calbiochem-Novabiochem) alone or 5 mg/L Act D plus 0.3 μmol/L doxorubicin, in the presence or absence of 20 μmol/L probucol (Sigma), for varying periods of time. Cells were collected, and total RNA was isolated. ANP and BNP mRNAs were detected and normalized for expression of the GAPDH transcript as described above. Results are expressed as percent of this normalized ratio at zero time in the absence of additions.

DNA Transfection and CAT and Luciferase Assays

Cells were transfected on the day of isolation with 20 μg of -109 thymidine kinase (TK) promoter–driven chloramphenicol acetyl-transferase (CAT). 23 2 μg of -1595 human BNP (hBNP)-luciferase, 22 or 2 μg of -2593 human ANP (hANP)-luciferase (−2593 to +18 relative to the transcription start site in hANP gene, linked to luciferase reporter in pMG-1). Transient transfection was performed as described previously. 22 After 48 hours, media was changed to DMEM/SS that contained doxorubicin or other agents, as indicated, and the incubations were continued for varying periods of time. Cells were then collected, and lysates were generated as described previously. 22 Equal amounts of extract protein were processed for measurement of luciferase or CAT activity.

[H]Uridine Incorporation

Cells were cultured in DMEM/ECS in 24-well plates for 48 hours. At that point, all cells were placed in DMEM/SS and treated with 5 mg/L Act D for defined periods of time. To determine effects on new RNA synthesis, cells were pulsed with 2 μCi/L [5,6-3H]uridine (NEN Research Products) in minimal Eagle’s medium (MEM) with Earle’s balanced salt solution (EBSS) containing the same additives for the last 4 hours of the incubation. Cells were then washed 3 times with PBS and treated with 10% trichloroacetic acid for 30 minutes at 4°C. Cellular residues were rinsed in 95% ethanol, solubilized in 0.25N NaOH at 4°C for 2 hours, and then neutralized with 2.5 mol/L HCl/1 mol/L Tris HCl (pH 7.5). Incorporated radioactivity was determined by scintillation counting.

To assess nascent RNA stability, atrial cells were grown in DMEM/ECS in 24-well dishes for 48 hours. At that point, cells were pulsed with 2 μCi/L [3H]uridine in MEM/EBSS for 4 hours. After incubation, the media was discarded, and cells were washed 4 times with PBS and cultured in DMEM/SS containing 0.1 mmol/L unlabeled uridine, in the presence or absence of 5 mg/L Act D, 0.3 μmol/L doxorubicin, or 20 μmol/L probucol, for 4, 8, or 24 hours. At each time point, cells were washed 3 times with PBS, and [3H]uridine incorporation was assayed according to the protocol described above.

Statistical Analysis

Data are presented as mean ± SD. Statistical analysis was performed by using 1-way ANOVA and the Newman-Keuls test for significance.

Results

Doxorubicin is known to be toxic to cultured cardiac myocytes, particularly at higher concentrations. 23 Because we were interested in looking at gene expression in viable myocytes rather than decay of transcriptional activity in different time intervals. Total RNA was isolated from cells with the RNeasy mini kit (Qiagen, Inc) according to the instructions provided by the manufacturer. RNA (8 to 10 μg) was separated on a gel that contained 2.2% formaldehyde, transferred to a nitrocellulose filter, and hybridized with a 640-bp fragment of the rat BNP cDNA. The blots were subsequently stripped of probe and rehybridized with an 840-bp fragment of the rat ANP cDNA. To normalize the blots for differences in RNA loading and/or transfer to the membranes, the blots were stripped a second time and rehybridized with a 1.3-kb GAPDH cDNA probe. Autoradiographic signals were quantified by use of the NIH Image program. For measurement of mRNA stability, cells were cultured in DMEM/SS with 5 mg/L actinomycin D (Act D, Calbiochem-Novabiochem) alone or 5 mg/L Act D plus 0.3 μmol/L doxorubicin, in the presence or absence of 20 μmol/L probucol (Sigma), for varying periods of time. Cells were collected, and total RNA was isolated. ANP and BNP mRNAs were detected and normalized for expression of the GAPDH transcript as described above. Results are expressed as percent of this normalized ratio at zero time in the absence of additions.
Figure 1. Doxorubicin decreases BNP and ANP secretion in dose- and time-dependent fashion. Atrial cells were cultured for 48 hours. At that point, cells were placed in DMEM/SS and treated with different concentrations of doxorubicin for 24 hours (A) or 0.3 μmol/L doxorubicin for varying periods of time (B). Culture medium was collected and subjected to radioimmunoassay. Control activity was determined in parallel untreated cultures. For all subsequent experiments, doxorubicin was used at a maximal concentration of 0.3 μmol/L for periods as long as 24 hours (A) or 0.3 μmol/L doxorubicin for varying periods of time (B).

A

B

We next examined the ability of ICRF-187 (Pharmacia, Inc) to prevent the doxorubicin-dependent reduction in NP gene expression. As shown in Figure 4A and 4B, ICRF alone effected a modest increase in ANP mRNA levels. It led to an even more pronounced increase in those cultures treated with doxorubicin (ie, reversal of the doxorubicin-dependent reduction in ANP mRNA levels); however, ICRF had no effect on steady-state BNP mRNA levels, either in the presence or absence of doxorubicin. Preincubation of the cultures with ICRF for 2 hours did not amplify the effect on ANP mRNA levels; however, ICRF had no effect on BNP gene expression.

There was a similar ICRF-dependent increase in ANP gene promoter activity (Figure 4C), either in the presence or absence of doxorubicin, although the magnitude of the effect (ie, fold induction) was considerably larger in the presence of the drug. ICRF failed to restore BNP gene promoter activity after doxorubicin treatment, implying selectivity in the response. We assume that the increase in ANP gene promoter activity accounts, at least in part, for the recovery of ANP mRNA levels.

Similar analyses were performed by using the antioxidant probucol. Unlike ICRF, probucol alone had little effect on
either ANP or BNP mRNA levels (Figures 5A and 5B); however, it effected a near-complete recovery of doxorubicin-suppressed ANP transcript levels. The highest concentration of probucol effected only a modest increase in doxorubicin-suppressed BNP mRNA levels.

Probucol alone had no effect on either ANP or BNP gene promoter activity; however, like ICRF, it partially reversed doxorubicin-dependent suppression of the ANP, but not BNP, promoter (Figure 5C). The combination of ICRF and probucol together had no greater effect than that seen with probucol alone. It should be noted, however, that probucol, in the presence of doxorubicin, increased ANP gene promoter activity to only a fraction of that seen in the control cultures, whereas ANP mRNA levels approached those of the control cultures at higher probucol concentrations (Figure 5A), implying enhanced stability of the ANP transcript in the presence of the antioxidant.

To examine this latter question in greater detail, we used Act D to suspend RNA synthesis in our atrial myocyte cultures and followed the decay of ANP or BNP mRNA levels in the presence or absence of probucol. At the concentration used in the present study, Act D reduced \( [\text{H}] \)uridine incorporation by \( 95\% \) at 5 hours and by \( 99\% \) after 24 hours of incubation (data not shown), indicating adequate inhibition of transcription. As shown in Figures 6A and 6B, the half-life of the native ANP gene transcript is in the range of 24 hours, whereas that for the BNP transcript is \( \approx 4 \) to 5 hours, supporting the previously reported differential transcript stability for these 2 gene products.\(^{\text{24}}\) Of note, the inclusion of probucol in the incubation led to a significant stabilization of the ANP transcript (half-life \( >24 \) hours) but had no effect on the BNP transcript. In the presence of doxorubicin, BNP transcripts tended to be more stable than in the absence of the drug (Figures 6C and 6D). In this setting,
probucol had a modest stabilizing effect on the BNP mRNA. The ANP transcript half-life was not significantly affected by inclusion of doxorubicin; however, the addition of probucol led to enhanced stability of the transcript in both settings.

Collectively, these data indicate that probucol reverses doxorubicin-dependent suppression of ANP gene expression by increasing transcriptional activity of the gene (ie, by preventing doxorubicin-dependent suppression of ANP gene transcription) and by effecting a stabilization of the ANP mRNA in doxorubicin-independent fashion. Probucol failed to protect BNP gene transcription and afforded only a modest increase in transcript stability in the presence of doxorubicin. It is the latter effect, presumably, that accounts for the slight increase in steady-state BNP transcript levels seen in Figure 5.

To determine whether the effects of probucol and/or doxorubicin could be extrapolated to a more general RNA population in these cells, we pulsed cells with [3H]uridine for 4 hours, then washed them free of the label, and added unlabeled uridine (0.1 mmol/L) and Act D, in the presence or absence of doxorubicin and/or probucol, for different time intervals. Measurements of [3H]uridine incorporation at different time points provided information on the stability of mRNA sequences.

**Figure 3.** Dose- and time-dependent inhibition of BNP and ANP promoter activity by doxorubicin. Cells were transfected with 2 μg of −1595 hBNP-luciferase or −2593 hANP-luciferase reporter. After transfection, cells were cultured and treated as described in Figure 1. Cells were then collected for measurement of luciferase activity. Data presented were obtained from 4 independent experiments. Dose dependence of the doxorubicin effect is presented in A. The time course of the response is provided in B. +P<0.05, *P<0.01 vs control.

**Figure 4.** Effect of ICRF-187 on basal and doxorubicin-inhibited BNP and ANP mRNA levels and promoter activities. Cells were exposed to the indicated concentrations of ICRF, with or without 0.3 mmol/L doxorubicin (Dox), for 24 hours. In a single group, cells were preincubated with 6 mmol/L ICRF for 2 hours before the addition of 0.3 mmol/L Dox for 24 hours. BNP and ANP mRNAs were detected as described in Figure 2. Representative experiments are shown in A. Pooled data (n=4) are presented in B. In separate experiments, atrial cells were transfected with hBNP-luciferase and hANP-luciferase. Forty-eight hours after transfection, cells were treated as above, cultured for 24 hours, and harvested for luciferase assay. Data are derived from 5 independent experiments (C). #P<0.05, *P<0.01 vs control; +P<0.01 vs Dox alone.
different time points during the “chase” period provide assessments of the time-dependent stability of the nascently labeled RNA population. As shown in Figure 7, after suspension of RNA synthesis by Act D, 3H-RNA levels decayed by \( \sim 50\% \) after 4 hours and by \( >75\% \) after 24 hours. Probucol alone had no effect on the level of \([3H]\)uridine incorporation but did promote a modest increment above that seen in the presence of Act D that was statistically significant at 8 and 24 hours, implying that probucol has a modest stabilizing effect on this newly synthesized RNA population. Doxorubicin treatment also led to a decrease in 3H-RNA levels over time, but this decrease was not additive with that produced by Act D, implying that doxorubicin, like Act D, acts predominantly at the level of RNA synthesis and has little additional destabilizing effect on existing transcripts. Probucol partially reversed the decrease in 3H-RNA levels seen with doxorubicin treatment at 8 and 24 hours, presumably reflecting a combination of mRNA stabilization with or without recovery of doxorubicin-suppressed RNA synthesis.

Discussion

The present study provides the first report of selective inhibition of NP gene expression by doxorubicin in cardiac myocytes. This inhibition was demonstrated under conditions that did not affect the viability of the cell population under study. The important findings presented here are as follows: (1) ANP and BNP secretion, steady-state mRNA levels, and gene promoter activity are suppressed by doxorubicin treatment. Particularly noteworthy, BNP appears to be uniquely sensitive to the effects of this drug. (2) Both ICRF and probucol, putative antagonists of doxorubicin cardiotoxicity, restore steady-state levels of ANP mRNA to near control levels yet display a much more modest effect in reversing the inhibition of ANP gene promoter activity. (3) Neither ICRF nor probucol has a major impact on doxorubicin-dependent reductions in BNP mRNA levels or promoter activity. (4) Probucol appears to have a stabilizing effect on transcripts in atrial myocytes. This is most marked for the ANP gene transcript, less marked for a newly synthesized RNA population, and quite limited for the BNP transcript. (5) The inhibitory activity of doxorubicin appears to operate predominantly at the level of new RNA synthesis. There is no decrease in NP mRNA stability in the presence of doxorubicin; in fact, the BNP mRNA appears to be more stable in the presence of the drug.

It has been reported previously that doxorubicin targets the expression of specific genes in promoting its myocardiopathic effects. These include genes involved in sarcomeric function, like \( \alpha \)-actin, troponin I, and myosin light chain 2, as well as a number of genes involved in ATP production, including a heart- and muscle-specific isoform of ADP/ATP translocase, the Reiske iron-sulfur protein (a ubiquitously expressed electron transport chain component), and a muscle isozyme of phosphofructokinase. The precise molecular mechanism underlying this inhibition of gene transcription is unknown but has been suggested to be linked to a reduction of myoD activity; however, studies from Evans et al indicate that overexpression of Id, a suppressor of myoD activity, in cardiac myocytes does not result in significant inhibition.
suppression of the ANP gene promoter, implying that the mechanism underlying doxorubicin-dependent inhibition of this gene may be distinct from that governing inhibition of other myogenic proteins.

Our analysis identifies 2 cardiac-specific genes encoding secretory products (ANP and BNP) whose expression is inhibited by doxorubicin but suggests that there are striking differences in their relative sensitivity to the drug. These genes, which are positioned in proximity on the same chromosome, are both expressed in the heart, albeit with different chamber specificity.\textsuperscript{12} They are both activated by similar provocative stimuli, typically those associated with cardiac hypertrophy.\textsuperscript{13–16} They encode peptides that bind and activate the same receptor in target tissues at the periphery, and they display very similar physiological properties in laboratory animals and humans. Despite these similarities, the sensitivity of BNP gene expression to doxorubicin inhibition is much greater than that seen with ANP. In addition, and equally important, the latter inhibition is fully reversible with antioxidant intervention, whereas BNP expression is almost completely unaffected. This indicates that there is something quite different about the mechanisms that underlie doxorubicin-induced suppression of these 2 genes and that the pro-oxidant hypothesis (see below), which seems to be the leading contender at present, cannot explain all of the effects of the drug, even at the level of transcription.

Plasma ANP and BNP levels are known to be increased in pathophysiological states associated with cardiac hypertrophy and failure. In fact, a number of recent studies have suggested that measurement of plasma levels of these peptides may prove useful in identifying the presence of early cardiac dysfunction.\textsuperscript{29} The role of plasma NP measurements in

Figure 6. Probucol (Prob) stabilizes ANP mRNA in atrial myocytes. Cells were exposed to 5 mg/L Act D, with or without 20 \( \mu \text{mol/L} \) Prob, for indicated time intervals. BNP and ANP transcript levels were detected by Northern blot analysis as described in Figure 2. Representative experiments are provided in A. Pooled data from 4 independent experiments are shown in B. In separate experiments, cells were treated with 5 mg/L Act D and 0.3 \( \mu \text{mol/L} \) Dox, with or without 20 \( \mu \text{mol/L} \) Prob, for varying periods of time. Representative autoradiographs are shown in C. Data from 3 independent experiments are presented in D. **\( P<0.05 \), *\( P<0.01 \) vs control; +\( P<0.05 \), #\( P<0.01 \) vs respective Act D alone or Act D+Dox groups.
assessing doxorubicin toxicity is less well defined. Bauch et al. showed that 6 of 16 pediatric patients treated with doxorubicin (45 mg/m² body surface area) showed elevations in plasma ANP levels 3 weeks after administration of the drug. In a series of animal studies, Bernardini et al. found that a single dose of doxorubicin (10 mg/kg IV) in normal female Wistar rats resulted in a significant decrease in plasma ANP levels (compared with vehicle-injected controls) 3 to 6 hours after administration of the drug, whereas rats subjected to a more chronic dosing regimen (3 mg/kg IV per week) displayed a significant increase in plasma ANP levels 21 and 31 days after administration of the drug. Thus, it would appear that the plasma ANP response to doxorubicin is biphasic with early suppression, perhaps reflecting acute myocardial injury, followed by a subsequent stimulation as cardiac hypertrophy/failure develops. Our findings, which show a clear reduction in ANP secretion and gene expression after doxorubicin treatment, are compatible with the acute response reported by Bernardini et al. The late elevation in ANP reported by both Bauch et al and Bernardini et al probably reflects the response of residual myocardium to the progressive decline in cardiac function that develops as the sequelae of the initial insult begins to accrue. The findings presented here also indicate that doxorubicin-induced suppression of BNP secretion/expression, which has not been reported previously, could prove useful as a highly sensitive (versus ANP) marker of acute drug toxicity.

As noted above, doxorubicin has been reported to have a number of toxic effects on the myocardial cell. These include pro-oxidant effects resulting in free radical generation, interference with calcium dynamics in the sarcoplasmic reticulum and plasma membrane, and direct inhibition of RNA synthesis and protein production due to intercalation in genomic DNA. Whereas the latter may be primarily respon-

sible for its tumorigenic properties, free radical generation appears to be more closely tied to its cardiac toxicity. The myocardium is particularly vulnerable, given the limitations of its endogenous antioxidant systems. Furthermore, both ICRF, a heavy metal chelator that severely constrains iron-dependent free radical generation in the myocardial cell, and probucol, a hypolipidemic agent with potent antioxidant properties, have been shown to reverse the cardiac toxicity of the anthracyclines in whole-animal and/or clinical studies. The inhibitory activity of doxorubicin at the level of ANP gene transcription appears to be tied to its pro-oxidant properties in that both ICRF and probucol at least partially reversed the inhibition. Nevertheless, under conditions that completely reversed the reduction of ANP mRNA levels, neither ICRF nor probucol effectively restored BNP gene expression. This may suggest that the BNP gene is sensitive to a level of oxidant stress that does not perturb ANP gene expression in these cells or that other mechanisms, not operative in the case of ANP, are involved in regulating levels of the BNP transcript.

In summary, the clinical use of the anthracycline doxorubicin is limited by its cardiotoxic effects. These are believed to be related to the pro-oxidant activity of the drug in the myocardial cell. The present study indicates that sublethal concentrations of doxorubicin selectively suppress NP gene expression in cultured atrial cardiac myocytes. However, expression of the ANP gene is less affected than is the expression of the BNP gene, and suppression of the latter appears less clearly tied to the free radical–generating properties of the drug. Assessment of NP gene expression, particularly BNP gene expression, could prove to be a highly sensitive marker of doxorubicin toxicity in the acute setting.

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References


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