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

Songcang Chen, Miklos Garami and David G. Gardner

Hypertension 1999;34;1223-1231
Hypertension is published by the American Heart Association. 7272 Greenville Avenue, Dallas, TX 72514
 Print ISSN: 0194-911X. Online ISSN: 1524-4563

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.[1] 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.[2]

The mechanisms that underlie the cardiotoxicity are only partially understood. Possible mechanisms include direct or indirect release of endogenous toxins (eg, histamine),[3] alterations in intracellular calcium homeostasis,[4] generation of free radicals that damage cellular membranes,[5,6] and intercalation of drug in the nuclear and mitochondrial genome, resulting in diminished RNA and protein synthesis.[7] 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.[8] Because the heart is relatively deficient in those enzymes responsible for clearing free radicals (ie, superoxide dismutase, catalase, and glutathione peroxidase),[6] the administration of doxorubicin may lead to significant lipid peroxidation and destruction of mitochondrial membranes.

Dexrazoxane (ICRF-187),[9] a chelator of intracellular iron, and probucol,[10] 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.[10] 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.[11] BNP is more uniformly distributed between atrial and ventricular myocardium.[12] 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,[13,14] whole-animal,[15] and clinical[16] models.

Bauch et al[17] reported elevations of plasma ANP in pediatric patients 3 to 5 weeks after doxorubicin treatment. A more detailed animal study from Bernardini et al[18] 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

Received May 13, 1999; first decision June 1, 1999; revision accepted July 15, 1999.

From the Metabolic Research Unit and Department of Medicine, University of California at San Francisco. The present address for M. Garami is Second Department of Pediatrics, Semmelweis University School of Medicine, Budapest, Hungary.

Correspondence to David G. Gardner, Box 0540, Metabolic Research Unit, University of California at San Francisco, San Francisco, CA 94143. E-mail gardner@itsa.ucsf.edu

Hypertension **is available at http://www.hypertensionaha.org**

Effect of Dox on Atrial Cell Viability

	% Control
Dox, μmol/L	
0	100±6.8
0.003	97.0±3.9
0.03	94.8±7.7
0.3	90.7±3.7
3	39.5±4.8*
Time, h	
0	100±5.3
3	94.1±8.7
6	96.4±5.8
12	94.8±7.4
24	89.3±4.9
48	35.5±4.6*

Values are mean±SD from 3 different experiments. Atrial myocytes were plated in 96-well dishes at a density of 3×10^4 cells/well. After cells were cultured for 48 hours, they were treated with Dox at the indicated concentration for 24 hours or 0.3 μmol/L Dox for the time indicated. Cell viability based on absorbance (490 nm) was measured by cell proliferation assay.

*$P<0.01$ vs control.

inhibits the secretion and expression of both genes, albeit with different levels of effectiveness.

Methods

Cell Culture

Atrial myocyte–enriched cultures were generated from the upper one third of 1-day-old neonatal rat hearts by alternate cycles of trypsin digestion and mechanical disruption as previously described.[19] Cells were cultured in DMEM containing 10% enriched calf serum (ECS, Gemini Bioproducts), 2 mmol/L glutamine, 1×10^{-5} U/L penicillin, and 100 g/L streptomycin for 48 hours before switching to serum substitute (SS) medium.[20]

Cell Viability Bioassay

Cell viability in the atrial myocyte cultures was measured by use of the Celltiter 96 AQ_{ueous} Non-Radioactive Cell Proliferation Assay kit (Promega). Atrial cells were cultured in 96-well plates at a density of 3×10^4 cells per well for 48 hours before changing to DMEM/SS. Cells were then treated with different concentrations of doxorubicin for 24 hours or 0.3 μmol/L doxorubicin (Sigma Chemical Co) for variable periods of time. Tetrazolium compound was added to each well according to the instructions provided by Promega and then incubated for 2 to 4 hours at 37°C in a humidified 5% CO_2 atmosphere. Absorbance at 490 nm was recorded by an ELISA plate reader (Microplate, EL 310, Bio-Tek Instruments).

Radioimmunoassay

Atrial cells were plated in 24-well dishes at a density of 10^5 cells per well and cultured in DMEM/ECS for 48 hours. At that point, all cells were placed in DMEM/SS and treated with different concentrations of doxorubicin for 24 hours or 0.3 μmol/L doxorubicin for varying periods of time. The culture medium of each well was collected, centrifuged to remove cellular debris, and frozen at −70°C until assayed. Radioimmunoassay was performed with the use of a commercial kit (Peninsula Laboratories) according to the instructions provided by the manufacturer.

Northern Blot Analysis

Atrial cells were cultured in DMEM/ECS for 48 hours, changed to DMEM/SS, and then challenged with the agents indicated for 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-Novachem) 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 acetyltransferase (CAT),[21] 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 mCi/L [5,6-^{3}H]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 mCi/L [^{3}H]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 [^{3}H]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

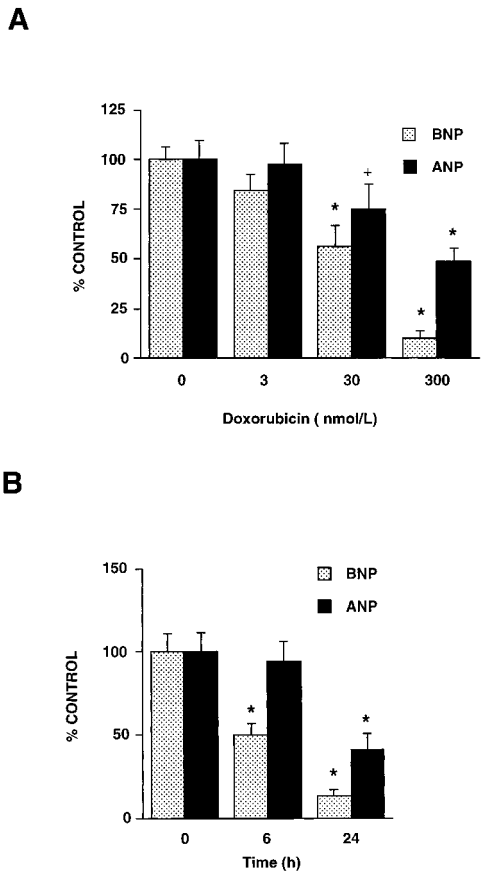

Figure 1. Doxorubicin decreases BNP and ANP secretion in dose- and time-dependent fashion. Atrial cells were cultured for 48 hours. At that point, cells were placed in DMEM/SS and treated with different concentrations of doxorubicin for 24 hours (A) or 0.3 μmol/L doxorubicin for varying periods of time (B). Culture medium was collected and subjected to radioimmunoassay. Control activity was determined in parallel untreated cultures in this figure (BNP, 7.3±0.68 ng/L per hour; ANP, 32.9±4.1 ng/L per hour) and in all subsequent figures. Data represent the mean±SD from 3 different experiments. $+P<0.05$, $*P<0.01$ vs control.

premorbid cells, we examined the viability of cells in our atrial myocyte cultures as a function of doxorubicin concentration and duration of treatment. As shown in the Table, cell viability was well preserved with doses of doxorubicin as high as 0.3 μmol/L for periods as long as 24 hours (≈90% viability), whereas higher concentrations of doxorubicin (3 μmol/L) or longer exposure times (48 hours) led to a precipitous decrease in the number of viable cells in the cultures. For all subsequent experiments, doxorubicin was used at a maximal concentration of 0.3 μmol/L for no longer than 24 hours. This dose range and duration of exposure have been shown to selectively inhibit muscle gene expression in the cardiac myocytes.[23]

We examined the effect of doxorubicin on the secretion of immunoreactive (ir) ANP and BNP in these cultures. As shown in Figure 1A, doxorubicin effected a dose-dependent decrease in irNP release from the atrial myocytes. Noteworthy, however, the inhibition was considerably more effective for irBNP (maximal inhibition, 90% at 0.3 μmol/L doxorubicin) than for irANP (maximal inhibition, 50% under the same conditions), implying greater sensitivity of BNP versus ANP to the inhibitory effects of this drug. Differential sensitivity to doxorubicin was also seen in an abbreviated time-course experiment presented in Figure 1B. Inhibition of irBNP secretion was first seen at 6 hours (versus 24 hours for irANP), whereas maximal inhibition after 24 hours of exposure to the drug was ≈90% for irBNP and ≈60% for irANP.

Similar findings were noted at the level of NP gene expression. As shown in Figures 2A and 2B, steady-state levels of BNP mRNA were considerably more sensitive to the inhibitory effects of doxorubicin than were those of ANP. Maximal inhibition (ie, that seen at 0.3 μmol/L doxorubicin) was once again ≈90% for BNP and ≈60% for ANP. Levels of the GAPDH mRNA were unaffected by treatment with the drug. The kinetics of the fall in NP mRNA levels were similar to those seen at the level of secretion (Figures 2C and 2D). BNP mRNA levels were reduced by 50% after as little as 6 hours of exposure to doxorubicin and were near maximally suppressed after 24 hours, whereas inhibition of ANP mRNA levels (≈60% inhibition) was seen only after 24 hours of drug exposure.

The inhibition of steady-state NP gene transcript levels was mirrored at the level of promoter activity. Doxorubicin treatment of atrial myocytes transfected with either ANP or BNP gene promoter–driven luciferase reporters resulted in a dose-dependent (Figure 3A) and time-dependent (Figure 3B) decrease in reporter activity, implying that the inhibitory effects of doxorubicin operate, at least in part, at a transcriptional locus. These effects are not exerted on all promoters. Doxorubicin treatment of atrial myocytes transfected with a TK promoter–driven CAT reporter did not result in a significant reduction in reporter activity (data not shown). Noteworthy, the effects of the drug on ANP and BNP promoter activity were much more equivalent than the effects of the drug on their respective mRNA levels.

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 when those cultures were compared with samples to which doxorubicin and ICRF had been added simultaneously, nor did it restore 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

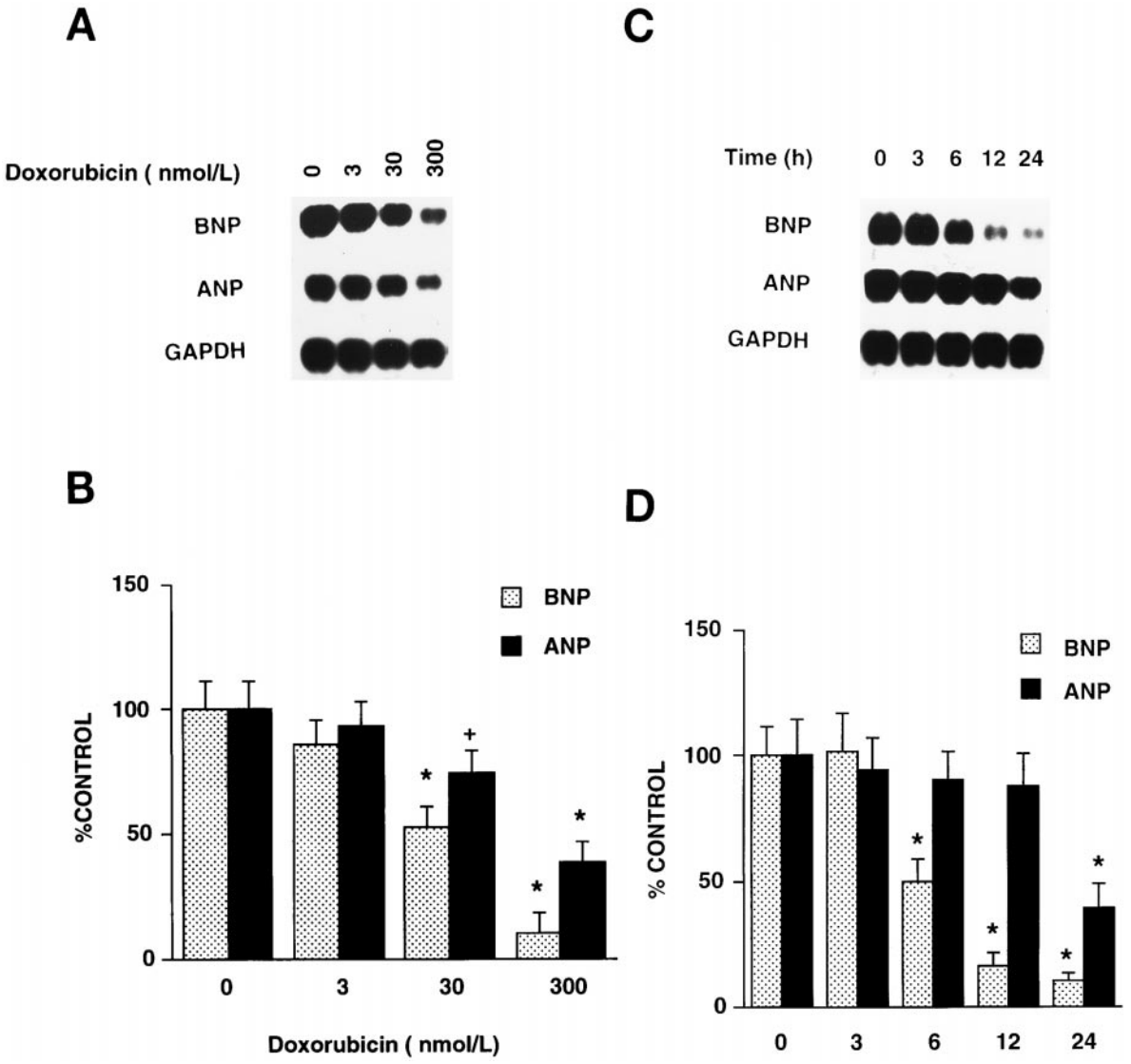

Figure 2. Doxorubicin effects a dose- and time-dependent reduction in both BNP and ANP mRNA levels. Cells were cultured and treated as described in Figure 1. Total RNA was isolated from cells. RNA (8 to 10 μg) was size-fractionated, transferred to a nitrocellulose filter, and sequentially blot-hybridized with radiolabeled cDNAs for BNP, ANP, and GAPDH. Representative experiments are presented in A and C. Pooled data (n=4) are shown as normalized BNP/GAPDH or ANP/GAPDH mRNA ratios in B and D. $+P<0.05$, $*P<0.01$ vs control.

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 [^{3}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 ≈4 to 5 hours, supporting the previously reported differential transcript stability for these 2 gene products.[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,

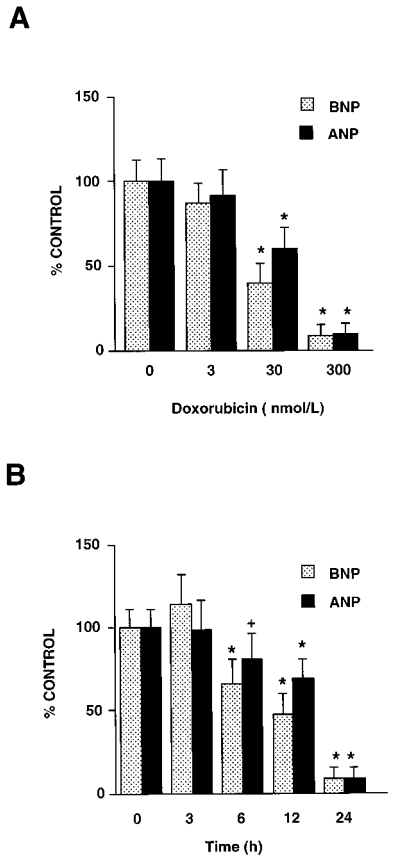

Figure 3. Dose- and time-dependent inhibition of BNP and ANP promoter activity by doxorubicin. Cells were transfected with 2 μg of −1595 hBNP- luciferase or −2593 hANP- luciferase reporter. After transfection, cells were cultured and treated as described in Figure 1. Cells were then collected for measurement 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.

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 [^{3}H]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 [^{3}H]uridine incorporation at dif-

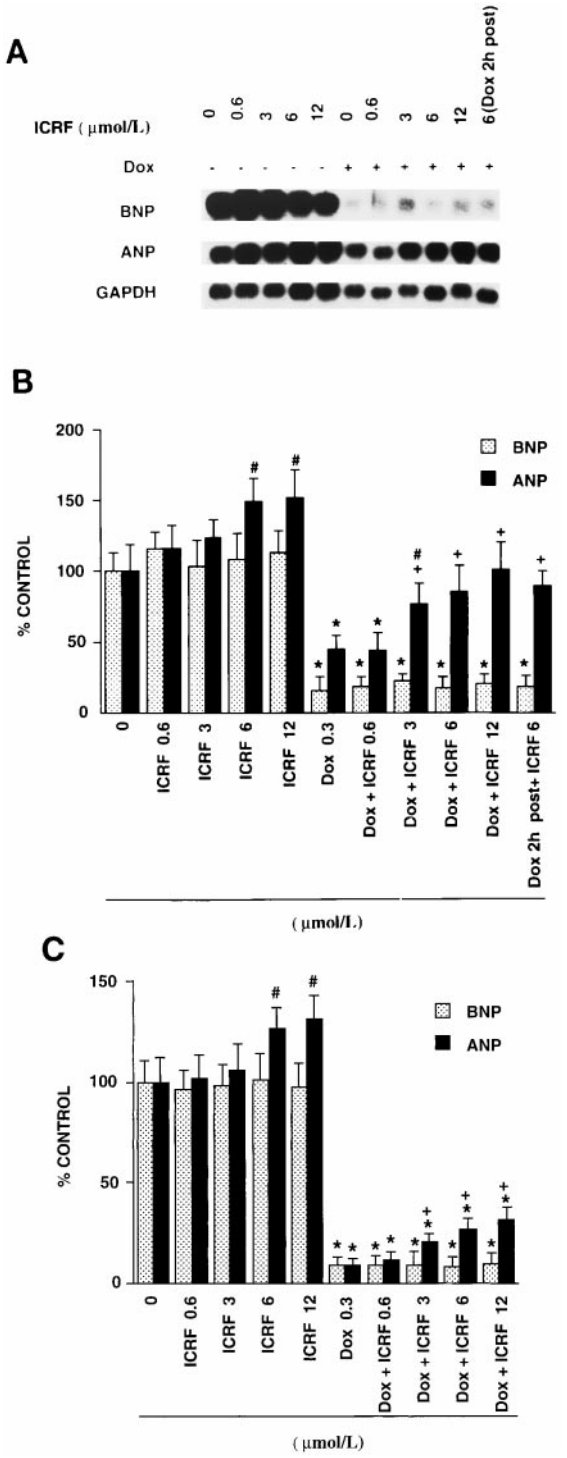

Figure 4. Effect of ICRF-187 on basal and doxorubicin-inhibited BNP and ANP mRNA levels and promoter activities. Cells were exposed to the indicated concentrations of ICRF, with or without 0.3 μmol/L doxorubicin (Dox), for 24 hours. In a single group, cells were preincubated with 6 μmol/L ICRF for 2 hours before the addition of 0.3 μmol/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.

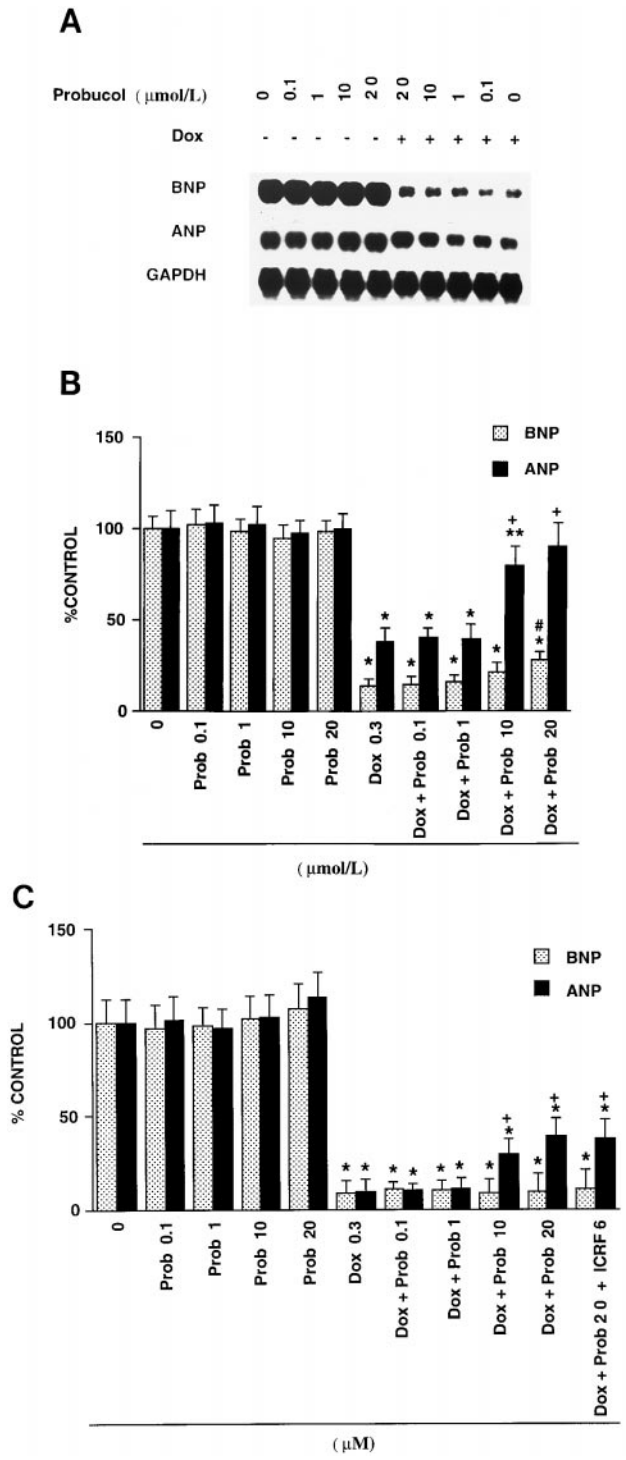

Figure 5. Effect of probucol (Prob) on basal and Dox-inhibited BNP and ANP transcript levels and promoter activities. Cells were treated with the indicated concentrations of probucol, with or without 0.3 μmol/L Dox, for 24 hours. BNP and ANP mRNAs were measured as described in Figure 2. Representative autoradiographs are shown in A. Pooled data from 3 independent experiments are presented in B. In separate experiments, cells were transfected with hANP-luciferase or hBNP-luciferase. Forty-eight hours later, cells were incubated with probucol in the presence or absence of Dox for 24 hours before measurement of luciferase activity. Pooled data are presented from 4 separate experiments (C). $^{**}P<0.05$, $^{*}P<0.01$ vs control; $\#P<0.05$, $+P<0.01$ vs Dox alone.

ferent 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, ^{3}H-RNA levels decayed by ≈50% after 4 hours and by >75% after 24 hours. Probucol alone had no effect on the level of [^{3}H]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 ^{3}H-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 ^{3}H-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,[23,25] like α-actin, troponin I, and myosin light chain 2, as well as a number of genes involved in ATP production,[26] 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[27]; however, studies from Evans et al[28] indicate that overexpression of Id, a suppressor of myoD activity, in cardiac myocytes does not result in significant

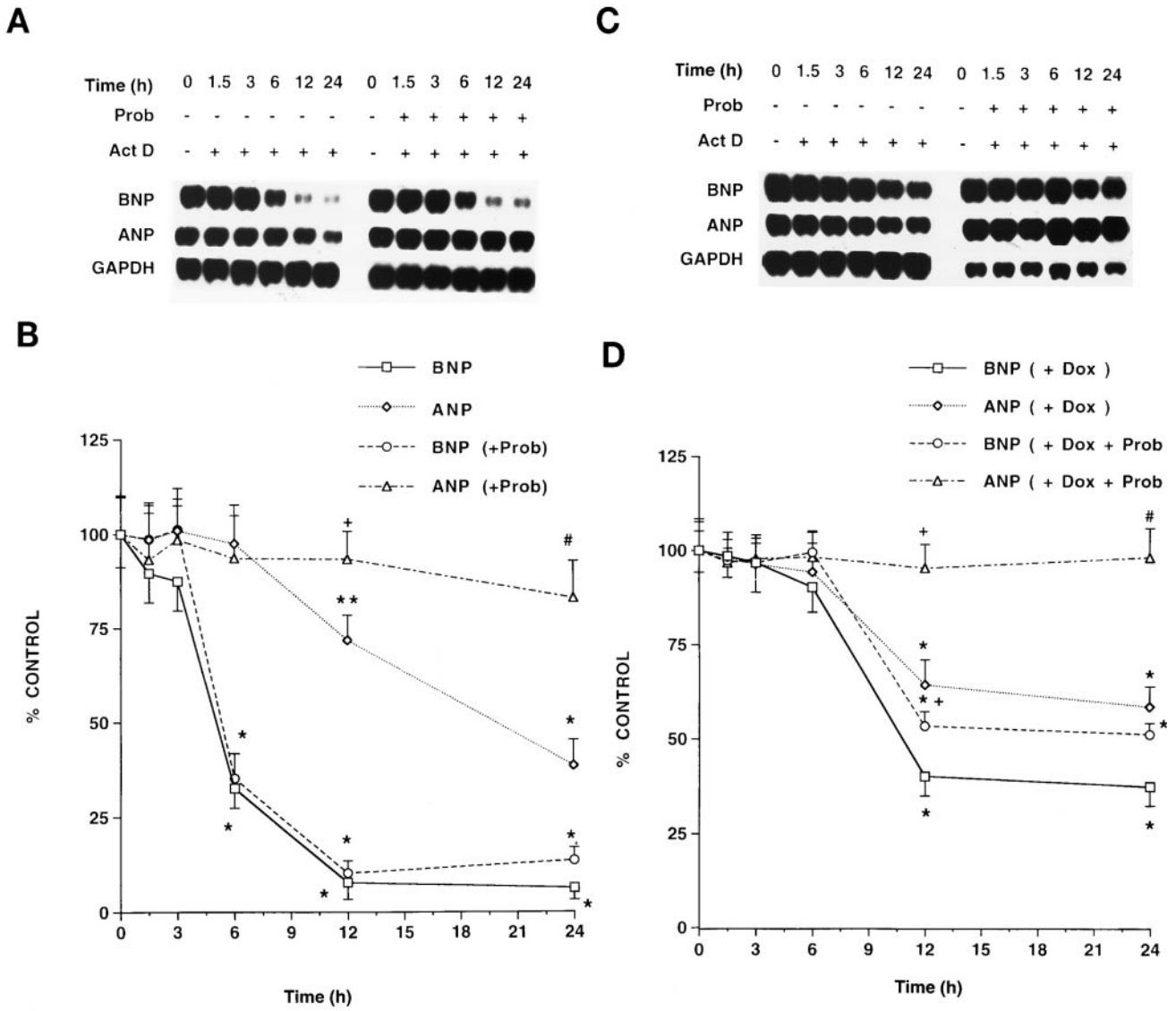

Figure 6. Probucol (Prob) stabilizes ANP mRNA in atrial myocytes. Cells were exposed to 5 mg/L Act D, with or without 20 μmol/L Prob, for indicated time intervals. BNP and ANP transcript levels were detected by Northern blot analysis as described in Figure 2. Representative experiments are provided in 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 μmol/L Dox, with or without 20 μmol/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.

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.[12] They are both activated by similar provocative stimuli, typically those associated with cardiac hypertrophy.[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.[29] The role of plasma NP measurements in

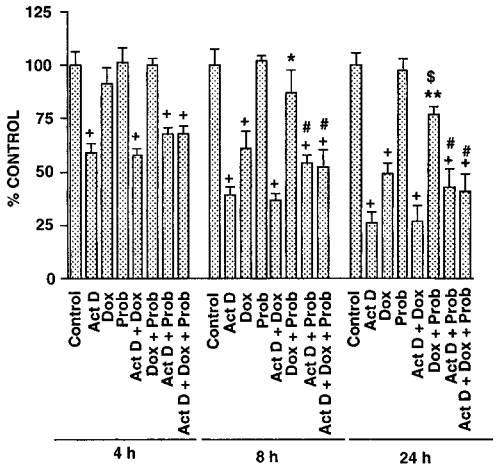

Figure 7. Effect of Prob on stability of newly synthesized RNA. Cells were pulsed for 4 hours with 2 μCi/mL [^{3}H]uridine in MEM/EBSS. At that point, cells were washed 4 times with PBS and then cultured in serum-free DMEM containing 0.1 μmol/L unlabeled uridine and, where indicated, 5 mg/L Act D, 0.3 μmol/L Dox, and/or 20 μmol/L Prob for the times indicated. [^{3}H]Uridine incorporation into RNA was assayed as described in Methods. Data are derived from 4 independent experiments. \$$P<0.05$, +$P<0.01$ vs control; *$P<0.05$, **$P<0.01$ vs Dox alone; and #$P<0.05$ vs Act D or Act D plus Dox.

assessing doxorubicin toxicity is less well defined. Bauch et al[17] showed that 6 of 16 pediatric patients treated with doxorubicin (45 mg/m^2 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[18] 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 myocyte toxicity, 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 Bausch 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,[5,6] interference with calcium dynamics in the sarcoplasmic reticulum and plasma membrane,[4] and direct inhibition of RNA synthesis and protein production due to intercalation in genomic DNA.[7] Whereas the latter may be primarily responsible for its tumoricidal 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.[6] 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 anthracycline in whole-animal[9,10] and/or clinical[30] 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.

Acknowledgments

This study was supported by grant HL-35753 from NIH. The authors are grateful to Karl Nakamura for preparation of the cells.

References

1. Hortobagyi GN. Anthracyclines in the treatment of cancer. *Drugs*. 1997; 54(suppl 4):1–7.
2. Ganz WI, Sridhar KS, Ganz SS, Gonzalez R, Chakko S, Serafini A. Review of tests for monitoring doxorubicin-induced cardiomyopathy. *Oncology*. 1996;53:461–470.
3. Bristow MR, Kantrowitz NE, Harrison WD, Minobe WA, Sageman WS, Billingham ME. Mediation of subacute anthracycline cardiotoxicity in rabbits by cardiac histamine release. *J Cardiovasc Pharmacol*. 1983;5: 913–919.
4. Kim DH, Landry AB, Lee YS, Katz AM. Doxorubicin-induced calcium release from cardiac sarcoplasmic reticulum vesicles. *J Mol Cell Cardiol*. 1989;21:433–436.
5. Keizer HG, Pinedo HM, Schuurhuis GJ, Joenje H. Doxorubicin (adriamycin): a critical review of free radical-dependent mechanisms of cytotoxicity. *Pharmacol Ther*. 1990;47:219–231.
6. Doroshow JH, Locker GY, Myers CE. Enzymatic defenses of the mouse heart against reactive oxygen metabolites: alterations produced by doxorubicin. *J Clin Invest*. 1980;65:128–135.
7. Zahringer J, Hofling B, Raum W, Kandolph R. Effect of adriamycin on the polyribosome and messenger-RNA content of rat heart muscle. *Biochem Biophys Acta*. 1980;608:315–323.
8. Seifert CF, Nesser ME, Thompson DF. Dexrazoxane in the prevention of doxorubicin-induced cardiotoxicity. *Ann Pharmacother*. 1994;28: 1063–1072.
9. Hershko C, Pinson A, Link G. Prevention of anthracycline cardiotoxicity by iron chelation. *Acta Haematol*. 1996;95:87–92.

10. Siveski-Iliskovic N, Kaul N, Singal PK. Probucol promotes endogenous antioxidants and provides protection against adriamycin-induced cardiomyopathy in rats. *Circulation*. 1994;89:2829–2835.
11. Gardner DG, Deschepper CF, Ganong WF, Hane S, Fiddes J, Baxter JD, Lewicki J. Extra-atrial expression of the gene for atrial natriuretic factor. *Proc Natl Acad Sci U S A*. 1986;83:6697–6701.
12. Gerbes AL, Dagnino L, Nguyen T, Nemer M. Transcription of brain natriuretic peptide and atrial natriuretic peptide genes in human tissues. *J Clin Endocrinol Metab*. 1994;78:1307–1311.
13. Sadoshima J, Izumo S. Molecular characterization of angiotensin II–induced hypertrophy of cardiac myocytes and hyperplasia of cardiac fibroblasts: critical role of the AT1 receptor subtype. *Circ Res*. 1993;73:413–423.
14. Nakagawa O, Ogawa Y, Itoh, H, Suga S, Komatsu Y, Kishimoto I, Nishino K, Yoshimasa T, Nakao K. Rapid transcriptional activation and early mRNA turnover of brain natriuretic peptide in cardiocyte hypertrophy: evidence for brain natriuretic peptide as an 'emergency' cardiac hormone against ventricular overload. *J Clin Invest*. 1995;96:1280–1287.
15. Tamura N, Ogawa Y, Itoh H, Arai H, Suga S, Nakagawa O, Komatsu Y, Kishimoto I, Takaya K, Yoshimasa T, Shiono S, Nakao K. Molecular cloning of hamster brain and atrial natriuretic peptide cDNAs: cardiomyopathic hamsters are useful models for brain and atrial natriuretic peptides. *J Clin Invest*. 1994;94:1059–1068.
16. Hasegawa K, Fujiwara H, Doyama K, Miyamae M, Fujiwara T, Suga S, Mukoyama M, Nakao K, Imura H, Sasayama S. Ventricular expression of brain natriuretic peptide in hypertrophic cardiomyopathy. *Circulation*. 1993;88:372–380.
17. Bauch M, Ester A, Kimura B, Victorica B, Kedar A, Phillips MI. Atrial natriuretic peptide as a marker for doxorubicin-induced cardiotoxic effects. *Cancer*. 1992;69:1492–1497.
18. Bernardini N, Agen C, Favilla S, Danesi R, Tacca MD. Doxorubicin cardiotoxicity is associated with alterations of plasma levels of atrial natriuretic factor. *J Endocrinol Invest*. 1992;15:79–84.
19. Wu JP, Lapointe MC, West BL, Gardner DG. Tissue-specific determinants of human atrial natriuretic factor gene expression in cardiac tissue. *J Biol Chem*. 1989;264:6472–6479.
20. Bauer RF, Arthur LO, Fine DL. Propagation of mouse mammary tumor cell lines and production of mouse mammary tumor virus in serum free medium. *In Vitro*. 1976;12:558–563.
21. McKnight SL, Kingsbury R. Transcriptional control signals of a eukaryotic protein-coding gene. *Science*. 1982;217:316–324.
22. Lapointe MC, Wu G, Garami M, Yang XP, Gardner DG. Tissue-specific expression of the human brain natriuretic peptide gene in cardiac myocytes. *Hypertension*. 1996;27:715–722.
23. Ito H, Miller SC, Billingham ME, Akimoto H, Torti SV, Wade R, Gahlmann R, Lyons G, Kedes L, Torti FM. Doxorubicin selectively inhibits muscle gene expression in cardiac muscle cells in vivo and in vitro. *Proc Natl Acad Sci U S A*. 1990;87:4275–4279.
24. Hanford DS, Glembotski CC. Stabilization of the B-type natriuretic peptide mRNA in cardiac myocytes by alpha-adrenergic receptor activation: potential roles for protein kinase C and mitogen-activated protein kinase. *Mol Endocrinol*. 1996;10:1719–1727.
25. Kurabayashi M, Jeyaseelan R, Kedes L. Antineoplastic agent doxorubicin inhibits myogenic differentiation of C2 myoblasts. *J Biol Chem*. 1993;268:5524–5529.
26. Jeyaseelan R, Poizat C, Wu H-Y, Kedes L. Molecular mechanisms of doxorubicin-induced cardiomyopathy. *J Biol Chem*. 1997;272:5828–5832.
27. Kurabayashi M, Jeyaseelan R, Kedes L. Doxorubicin represses the function of the myogenic helix-loop-helix transcription factor MyoD. *J Biol Chem*. 1994;269:6031–6039.
28. Evans SM, Walsh BA, Newton CB, Thorburn JS, Gardner PD, van Bilsen M. Potential role of helix-loop-helix proteins in cardiac gene expression. *Circ Res*. 1993;73:569–578.
29. Yamamoto K, Burnett JC Jr, Jougasaki M, Nishimura RA, Bailey KR, Saito Y. Superiority of brain natriuretic peptide as a hormonal marker of ventricular systolic and diastolic dysfunction and ventricular hypertrophy. *Hypertension*. 1996;28:988–994.
30. Swain SM, Whaley FS, Gerber MC, Weisberg S, York M, Spicer D, Jones SE, Wadler S, Desai A, Vogel C, Speyer J, Mittelman A, Reddy S, Pendergrass K, Velez-Garcia E, Ewer MS, Bianchine JR, Gams RA. Cardioprotection with dexrazoxane for doxorubicin-containing therapy in advanced breast cancer. *J Clin Oncol*. 1997;15:1318–1332.