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Abstract: To evaluate the mechanisms and the impact of the ACE-inhibitor perindopril (P) in a model of doxorubicin (D)- induced cardiotoxicity, male Wistar rats received D (1mg/kg/d, i.p. for 10 days), P (2 mg/kg/d by gavage from day 1 to day 18), D (for 10 days) + P (for 18 days) or saline.

D decreased systolic blood pressure, body and heart weights. Left ventricular diastolic diameter was increased by D ($p<0.05$) but it was not attenuated by P. D decreased plasma vitamin C ($p<0.05$) and increased the ascorbyl radical/vitamin C ratio ($p<0.05$). This ratio was attenuated by P ($p<0.05$). No difference was found among groups in cTnI, BNP concentrations and tissue oxidative stress (OS). Myocardial MCP-1 expression was higher in the D group. Cardiac kinin receptor (B_1R and B_2R) expression was not affected by D yet binding sites for B_2R and B_1R were increased in D+P and P groups, respectively ($p<0.05$).

In conclusion, D induced cardiac functional alterations, inflammation and plasma OS whereas tissue OS, and cardiac kinin receptors expression were not modified. P did not improve cardiac performance, but modulated kinin receptor expression and enhanced antioxidant defense.

INTRODUCTION

Anthracyclines are very effective broad-spectrum chemotherapeutic agents, but their use is restricted because of their acute, sub-acute and chronic cardiotoxicity. [1] Cardiac injuries are asymptomatic for a long period and can appear months even years after the last injection of the drug. The main consequence is dilated cardiomyopathy, which induces congestive heart failure and can thus be life-threatening. [2] Some risk factors of cardiac injury due to anthracycline have been described. These include age, underlying heart disease, doses of chemotherapy agents and previous irradiation. The mechanisms responsible for this cardiomyopathy are multifactorial, and it is now well known that oxidative stress plays an important role. [3, 4] The anthraquinone nucleus of anthracycline is reversibly converted to a free radical semiquinone in the presence of molecular oxygen leading to cellular damage 1. Cardiac injury is also associated with apoptosis, [5, 6] immune reactions, [7] genetic alterations [8] and the production of alcohol metabolites from anthracyclines. [9] All of these factors induce modifications in calcium homeostasis and the inflammatory state. This inflammatory context associated with tissue injury is also responsible for B_1 and B_2 kinin receptors (B_1R and B_2R) modulation. [10] Because alterations in the kallikrein-kinin system may contribute to the pathogenesis of heart failure, [11] one can wonder if changes in kinin receptors expression can also be involved in anthracycline cardiotoxicity. In animal studies, the use of antioxidants successfully limited this cardiotoxicity [12-16] whereas in human trials they failed to improve the prognosis. However, in clinical practice, it has been reported that dexrazoxane (an iron chelator) or anthracycline analogs are able to limit the above-mentioned harmful effects. Liposomal encapsulation and prolonged continuous infusion instead of bolus delivery have also been proposed, but neither was entirely satisfactory. [17-19] Nowadays, heart failure induced by anthracycline is treated with conventional drugs such as β blockers, aldosterone receptor

antagonists and angiotensin-1 converting enzyme inhibitors (ACEIs), all of which have proven their efficacy in the treatment of heart failure. [20]

A recent study has shown that ACEIs seem to effectively reduce anthracycline cardiotoxicity in adult patients who had received high doses of these chemotherapeutic agents. [21] ACEIs could limit left ventricular dilation and reduced the ejection fraction. Thus, given that free radical species are the major agents of anthracycline cardiotoxicity, we decided to assess the evolution of oxidative stress generated by doxorubicin injections in rats treated with an ACE inhibitor. The present study was undertaken to investigate the early pathophysiologic mechanisms of doxorubicin cardiotoxicity: evaluating oxidative stress status, inflammation and the level of bradykinin receptors expression. In this context we have studied the impact of an ACEI (perindopril) on these mechanisms.

MATERIALS AND METHODS

Preparation of a rat model of doxorubicin-induced cardiomyopathy.

Forty seven male Wistar rats (weight 300-390 g at the beginning of the experiment) were used in this study. The local ethics committee approved the experimental protocol and the investigators complied with authorization 6006 from the French Government, which complies with the Guide for the Care and use of Laboratory Animals published by US National Institutes for Health. Four groups were made up.

In the control group, the rats (n = 11) received saline: 1 ml/kg/d intraperitoneally (i.p.) from day 0 to day 10 and 2 ml/kg *per os (p.o.)* daily from day 0 to day 17.

In the doxorubicin-treated group (D), the rats (n = 12) were treated with doxorubicin (Pfizer, Paris, France) 1 mg/kg/d i.p. from day 0 to day 10 and saline *p.o.* as were controls. A total dose of 10 mg/kg was administered over the 10-day period.

In the perindopril group (P), the rats (n = 12) received perindopril (Servier, Paris, France) 2 mg/kg/d *p.o.* from day 0 to day 17 and saline i.p. as were controls.

The last group (n = 12) was treated with both doxorubicin and perindopril (D+P) at the same dose and over the same period as the two previous groups.

At day 18, the rats were anesthetized (sodium thiopental, 60 mg/kg i.p.) and put down. Just after abdominal incision, heparin (500 UI/kg) was injected into the inferior vein cava. Blood was taken and centrifuged (2000 G, 5 min) to obtain the plasma, which was immediately frozen. The heart was excised; the ventricles were cut into 4 segments and both heart segments and aortas were frozen in liquid nitrogen at – 80°C.

Heart function parameters

Body weight was monitored every day until the end of the study. Systolic blood pressure was measured every 3 days by the tail cuff method (LE 5007 Pressure Meter, Bioseb) throughout the protocol. On day 0 and 18, left ventricular performance was evaluated by echocardiography using an SSD 900 with a 7.5-MHz transducer (ALOKA, DMV Imaging, Cergy, France). Left ventricular end-systolic (LVSd) and diastolic (LVDd) diameters were measured, and fractional shortening (% FS) was calculated by the formula:

$$\% FS = [(LVDd - LVSd)/LVDd] \times 100.$$

Plasma evaluation.

- cTnI (cardiac Troponin I) plasma concentrations.

CTnI concentrations were determined with an established immunoassay (High sensitivity rat cardiac troponin-I Elisa kit, Lifediagnostics, USA) which uses two different affinity purified antibodies. As previously described, the first was used for solid phase immobilization and the second was conjugated with Horse Radish Peroxidase (HRP). The plasma was allowed to

react simultaneously with the two antibodies resulting in the cTnI sandwiched between the solid phase and HRP-conjugated antibodies. After removing unbound HRP-conjugated antibodies, an HRP substrate, which induced a blue color, was added. The addition of HCl changed the color to yellow. Using a spectrometer, the concentration of cTnI was calculated and was proportional to the absorbance at 450 nm. The results are expressed in ng/ml.

- Plasma concentrations of BNP (Brain Natriuretic Peptide).

BNP concentrations were determined with an immunoassay (EIA for BNP-32, Peninsula Laboratories, LLC, San Carlos, US). Briefly, peptide antibody was injected into specially treated wells. Rat plasma was added and incubated for 2 h with peptide antibody. Biotinylated peptide, which competes with the plasma samples for the antibody binding sites, was added. After one night of incubation, unbound biotinylated peptide was removed and streptavidinconjugate Horse Radish Peroxidase (SA-HRP) was added. An immobilized primary antibody/biotinylated peptide complex was then formed and excess SA-HRP was washed out. 3, 3', 5, 5'-tetramethylbenzidine dihydrochloride was added to react with the bound HRP, which induced a blue color. HCl was added to stop the reaction and the color changed to yellow. Then, absorbance was read at 450 nm and the concentrations of BNP were calculated. The results are expressed in ng/ml.

- Quantification of ascorbyl free radical by Electron Spin Resonance spectroscopy (ESR).

Thirty-five µl of plasma was put into a quartz capillary tube, which was then placed in an HS cavity for analysis at room temperature with a Bruker EMX band spectrometer. The following parameters were selected for optimal detection of ascorbyl radical: modulation frequency: 100 Hz, amplitude modulation: 0.8 G, microwave power: 40 Mw, microwave frequency: 8.5 GHz,

conversion time: 40 ms, time constant: 327 ms, scan time: 41 s, gain: 5.105, number of scans: 6. The height of the signal intensity was measured and expressed in AU.

Superoxide production in tissue

- Superoxide production evaluated by ESR spin probe.

Intracellular superoxide formation was determined by the measurement of 3-methoxycarbonyl- proxyl nitroxide ($CM^{\bullet}$) produced by the oxidation of the cell-permeable spin probe 1-hydroxy-3-methoxycarbonyl-2, 2, 5, 5-tetramethylpyrrolidine (CMH). Freshly isolated heart tissue was incubated in deferoxamine-chelated Krebs-HEPES solution containing CMH (200μM, Noxygen, Elzach, Germany), deferoxamine (25μM, Sigma, Saint Quentin Fallavier, France) and DETC (5μM) for 20 min at 37°C. [22] At the end of this incubation period, the tissue samples were immediately inserted into a polyethylene catheter and frozen in liquid nitrogen. Samples were analyzed in a liquid nitrogen ESR dewar. The acquisition parameters of the Bruker EMX spectrometer 100 K were as follows: modulation frequency: 100 kHz, amplitude modulation: 2 G, microwave power: 20 Mw, microwave frequency: 9.84 GHz, conversion time: 82 ms, time constant: 65 ms, scan time: 84 s, gain: 5.104, number of scans: 1. The height of the $CM^{\bullet}$ radical signal was measured, matched with heart sample weight and expressed in AU/g.

- Determination of thiobarbituric acid reactive substances (TBARs).

The level of lipid peroxide in the hearts was measured by a colorimetric reaction with thiobarbituric acid. The hearts were homogenized in ice-cold phosphate buffered saline (0.05 M, pH 7). Then, 1.5 ml of a solution containing trichloroacetic acid, thiobarbituric acid and hydrochloric acid was added. The color of the thiobarbituric acid pigment was developed in a water bath at 100 °C for 15 min. After cooling with ice to room temperature, 1 ml of 70%

trichloroacetic acid was added. After 1.5 h, the tubes were centrifuged and the colors of the TBARs layers were assessed at 553 nm. The absorbance values were compared with a standard curve. Total protein was determined according to the Bradford method. [23] The results are expressed in nmoles/g of proteins.

- Superoxide production by fluorescence histology.

In the presence of superoxide, ethidine, a fluorescent compound, is formed from dihydroethidium (DHE), which thus makes it possible to measure superoxide production. Frozen heart tissues were fixed for 10 min in acetone. Slides were incubated in a dark humidified chamber at room temperature with DHE (5 µmol/l) for 5 min. The slides were immediately analyzed with a computer-based digitizing image system (Microvision, France) using a fluorescent microscope (Eclipse 600, Nikon, France) connected to a video camera (TriCCD, Sony, France). Fluorescence was detected at 590 nm; the results are expressed in fluorescence intensity/nuclear number.

- Inflammatory state by immunohistochemistry.

Briefly, cryosections were fixed in acetone for 10 min. After washing in H_2O_2, monoclonal antibodies against MCP-1 (Santa Cruz, California, USA) were added to cryosections which were left overnight at 4°C. The slices were washed 3 times in PBS-Tween and incubated with peroxidase-conjugated secondary antibody (Dakocytomation, Denmark) for 1 h. This was followed by 3 additional washes in PBS-Tween and then 3-amino-9-ethylcarbazole, a Substrat-Chromogen (DakoCytomation) was added to the slices, which were counterstained with hemalum.

- B_1 and B_2 kinin receptors mRNA by real-time quantitative RT-PCR

Total cellular genomic RNA was extracted from homogenized hearts using the RNAeasy tissue mini kit, and a cDNA copy was synthesized using the QuantiTect Reverse Transcription kit, according to manufacturer's instructions (QIAGEN, Valencia, CA, USA). Quantitative real time PCR was performed using the Quantitect SYBR Green PCR (QIAGEN, Valencia, CA, USA) and the M×3000p real-time PCR system (Stratagene, La Jolla, CA, USA). The sequence of primers for amplification of rat kinin receptors and the reference standard was designed by Vector NTI and used:

	Sequences		Position	Gen Bank
B1 receptor Forward	5' GCA GCG CTT AAC CAT AGC GGA AAT 3'		367 - 391	NM_030851
B1 receptor Reverse	5' CCA GTT GAA ACG GTT CCC GAT GTT 3'		478 - 454	
B2 receptor Forward	5' AGG TGC TGA GGA ACA ACG AGA TGA 3'		882 - 906	NM_173100
B2 receptor Reverse	5' TCC AGG AAG GTG CTG ATC TGG AAA 3'		1014 - 990	
18s receptor Forward	5' TCA ACT TTC GAT GGT AGT CGC CGT 3'		363 - 386	X01117
18s receptor Reverse	5' TCC TTG GAT GTG GTA GCC GTT TCT 3'		470 - 447	

PCR conditions were: 95°C for 15 min, followed by 46 cycles at 94°C for 15 s, 60°C for 30 s and 72°C for 30 s. The cycle threshold (Ct) value represents the cycle number at which a fluorescent signal rises statistically above the background. [24] The relative quantification of gene expression was analyzed by the $2^{-\Delta Ct}$ method. [25]

- Density and distribution of kinin receptors by autoradiography

Radioligands for kinin B_1 receptor (3-(4 hydroxyphenyl) propionyl-desArg^9-D-Arg^0[Hyp^3, Thi^5, D-Tic^7, Oic^8]Bradykinin) and kinin B_2 receptor (3-(4 hydroxyphenyl)propionyl-DArg^0[Hyp^3, Thi^5, D-Tic^7, Oic^8]Bradykinin) were synthesized and kindly provided by Dr. Witold Neugebauer (Dept. Pharmacology, University of Sherbrooke, Sherbrooke, Quebec, Canada). They were iodinated by the chloramine T method. [26, 27] The heart was mounted in a gelatin block and serially cut into 20-µm thick coronal sections with a cryostat. Thus the sections were thaw-mounted on 0.2% gelatin-0.033% chromium potassium sulfate-coated slides and

kept at -80°C until use. On the day of experiments, sections were incubated at room temperature for 90 min in 25 mM PIPES-NH_4OH buffer (pH 7.4) containing: 1 mM 1,10-phenanthroline, 1 mM dithiothreitol, 0.014% bacitracin, 0.1 mM captopril, 0.2% bovine serum albumin (protease free) and 7.5 mM magnesium chloride in the presence of 200 pM of [^{125}I]-HPP-desArg^{10}-Hoe 140 (B_1 receptor radioligand) or 200 pM [^{125}I]-HPP-Hoe 140 (B_2 receptor radioligand). [28] Non-specific binding was determined in the presence of 1 μM of unlabelled antagonist: R715 (AcLys[D-βNal^7,Ile^8]des-Arg^9-BK) (B_1 receptor antagonist, [29]) or Hoe 140 (D-Arg[Hyp^3,Thi^5,D-Tic^7,Oic^8]-BK) (B_2 receptor antagonist, [30]). At the end of the incubation period, slides were transferred sequentially through four rinses of 4 min each in 25 mM PIPES (pH 7.4; 4 °C) dipped for 15 s in distilled water (4 °C) to remove the excess of salts, and then air-dried. Kodak Scientific Imaging Films BIOMAX TM MR® (Amersham Pharmacia Biotech Canada) were juxtaposed onto the slides in the presence of [^{125}I]-microscales and exposed at room temperature for 5 days (B_2 receptor) or 7 days (B_1 receptor). The films were developed (GBX developer) and fixed (GBX fixer). Autoradiograms were quantified by densitometry using an MCID™ image analysis system (Imaging Research, St. Catharines, Ont. Canada). A standard curve from [^{125}I]-a microscale was used to convert density levels into fentomoles per milligram of protein. [31] Specific binding was determined by subtracting values of nonspecific binding from that of total binding.

Statistical analysis

All data are expressed as means ± S.E.M. To compare the groups at day 18, statistical analyses were performed with a one-way analysis of variance (ANOVA) that was followed, if necessary, by a Newman Keuls test and by a Student's *t*-test for kinin receptor expression. The evolution of parameters during the protocol was analyzed with a two-way repeated measure ANOVA (SigmaStat). Significance was established at a value of $P < 0.05$.

RESULTS

Body weight

Whereas controls and perindopril-treated rats gained weight throughout the protocol, doxorubicin induced a significant loss of body weight ($p < 0.05$). Treatment with perindopril was not able to counter this loss of body weight. At the interruption of doxorubicin, the weight of both doxorubicin-treated groups began to increase (Figure 1A).

Heart weight, ratio heart weight to body weight

The heart weight of rats treated with doxorubicin, perindopril, and the combination of the two decreased significantly compared to that of saline controls (controls: 0.81 ± 0.02 g, D: 0.74 ± 0.02 g, P: 0.72 ± 0.01 g, D+P: 0.69 ± 0.01 g, $p<0.05$). The heart weight to body weight ratio (Figure 1B), which indicates the degree of cardiac hypertrophy, was significantly decreased by perindopril ($p<0.05$) in comparison to both controls and the doxorubicin treated group.

Systolic blood pressure

As shown in figure 2, arterial systolic blood pressure of controls was relatively stable at around 135 mmHg throughout the protocol. In contrast, doxorubicin caused a significant decrease in systolic blood pressure on day 6 and 9 in comparison with controls ($p < 0.05$). At the end of doxorubicin treatment, systolic blood pressure stabilized at values close to 130 mmHg. As expected, the blood pressure of rats treated with perindopril fell to around 110 mmHg from the sixth day to the end of the protocol ($p<0.05$ *vs.* controls). There was no cumulative effect of doxorubicin and perindopril on arterial blood pressure level in the D + P group.

Echocardiographic evaluation of left ventricular function

In each group, no differences in echocardiographic parameters were observed, between day 0 and day 11. By day 18, the end-diastolic left ventricular diameter (LVDd), assessed by echocardiography, in controls and in the rats which received perindopril was not modified, whereas that in animals treated with doxorubicin a significant increase in LVDd was observed ($p < 0.05$; Figure 3). Treatment with perindopril did not prevent this dilation. Concerning end-systolic left ventricular diameter and left ventricular fractional shortening, no differences were observed between values obtained at day 0 and day 18 in each group (data not shown).

Plasma cTnI and BNP concentrations

No differences were observed between the groups with regard to concentrations of plasma cTnI and BNP, assessed using ELISA kits (Table 1).

Plasma oxidative stress

Control and perindopril groups presented comparable plasma concentrations of ascorbate (Table 1). Treatment with doxorubicin led to a significant reduction in the concentration of this antioxidant ($p<0.05$ *vs.* controls and P). However, the D+P group did not show a significant fall in ascorbate concentration. The level of plasma oxidative stress was estimated by the ascorbyl radical to ascorbate ratio (Table 1). Compared to controls and perindopril-treated rats, a significant increase in this ratio was observed in the doxorubicin-treated group ($p<0.05$). Once again, treatment with perindopril attenuated the increase in the generation of plasma free radicals induced by doxorubicin (but it was not significant, p xxxxx).

Oxidative stress in tissue

No differences between the groups were found for cardiac oxidative stress with the different techniques used (TBARs, superoxide production by ESR, DHE) (Table 1).

Inflammatory status

In the doxorubicin and doxorubicin + perindopril groups, MCP-1 immunoreactivity was detected in coronary endothelium, myocardial interstitium and cardiomyocytes, yet it was only detected in coronary endothelium in the control and perindopril group (Figure 4).

B_1 and B_2 kinin receptors mRNA

Whereas cardiac B_2 receptor mRNA was not significantly different between control, D and D+P groups, an upward trend of B_2 receptor mRNA was observed in rats treated with P compared to controls ($p=0.074$). No differences in B_1 receptor mRNA was observed between the 4 groups (Figure 5A).

Density of B_1 and B_2 kinin receptors binding sites

An upward trend of B_2 receptor binding sites was observed in the D and P groups compared to controls. In the D+P group, this increase was significant when compared with controls ($p=0.012$). Concerning the B_1 receptor, cardiac binding sites increased in the D, P and D+P groups compared to controls, but this increase reached significance only in the P group ($p=0.041$) (Figure 5A and 5B).

DISCUSSION

The study of functional parameters showed a significant decrease in heart function in the D group which was not prevented by the association of perindopril. Heart weight reduction due to doxorubicin was described by Okumura *et al.* [32] and is probably due to apoptosis and slowed growth. No differences were observed between the control and D group concerning the heart to body weight ratio because both the heart weight and the body weight were decreased by doxorubicin to the same proportion so the ratio remained stable. This ratio, which reflects cardiac hypertrophy, was decreased in the P and D + P groups. This result supports the work of Ishimitsu *et al*., [33] and is explained by the use of perindopril, which induces a decrease in both angiotensin II, which has a hypertrophic effect on cardiovascular cells, and aldosterone which induces fibrosis in cardiovascular tissues. A significant decrease was observed in systolic blood pressure in the D and D + P groups. As previously described, [34] doxorubicin is responsible for a drop in arterial blood pressure. This drop cannot be explained by renal damage, which in the long term induces hypertension, [35] and according to Rabelo *et al.* [36] the sympathetic nervous system is not affected at this dose of doxorubicin. Thus, cardiac injury and deterioration in the general state of health may explain this fall. Regarding echocardiographic parameters, dilation of LVDd was observed in the D group. The increase in LVDd due to doxorubicin was reported by Bertinchant *et al.* [37] No significant changes were noted in the LVDs and in fractional shortening; the increase in LVDd may be the first step of cardiac remodeling. LVDd was also increased in the D + P group. Thus the addition of perindopril to doxorubicin did not prevent this dilation. Tokudome *et al.* [38] noted that cardiac remodeling was limited by Temocapril only after 12 weeks of treatment. Thus the absence of evidence of the efficacy of perindopril may be due to the short duration of the treatment.

Evaluation of plasma concentrations of cTnI and BNP showed no significant differences between the groups. Thus, no increase in plasmatic cTnI concentrations due to doxorubicin was found. This finding supports the work of Bertinchant [37] who considered different hypotheses to explain this result: minor myocyte damage, structural alterations of troponin or a decrease in troponin synthesis induced by anthracycline. Unlike the study of Kohyama *et al.* [39], which reported a significant increase in plasma concentration of BNP induced by anthracycline, we found no differences in plasma levels of BNP in our groups. However, Chen *et al.* [40] reported that doxorubicin inhibits BNP gene expression in cultured neonatal rat myocytes, and one can wonder if intra-peritoneal injections of doxorubicin also damage gene expression. Gene study may have helped us to interpret the plasma concentrations of cTnI and BNP.

Concerning oxidative stress, analysis of the plasma showed a decrease in the concentration of ascorbate, which indicated a decrease in the antioxidant defense by doxorubicin. This has also been observed in human studies [41] and can be explained by the decrease in hepatic ascorbate production and the increase in radical oxidative species as confirmed by the increase in the ascorbyl radical to ascorbate ratio. No significant differences were observed between the D + P group and controls with regard to levels of ascorbate and the ascorbyl radical to ascorbate ratio. The addition of perindopril to doxorubicin seems to have induced an increase in the plasma concentration of ascorbate and a decrease in the ascorbyl radical to ascorbate ratio. This supports the beneficial effects of perindopril in reducing anthracycline cardiotoxicity. Evaluation of oxidative stress in tissue by TBARs, superoxide production and DHE, did not show any significant differences between groups. This suggests that free radical production induced by anthracycline was compensated by the endogenous protective antioxidant defenses. These defenses may have already recovered seven days after the last injection of doxorubicin. No differences between the groups were found on tissue oxidative stress using

the CMH approach. Moreover, radical oxygen species are unstable, their lifespan is short and the damage they induce is not specific. Concerning the evaluation of superoxide production, using an ESR spin probe, few published studies have assessed levels of oxidative stress in tissues using CMH, and more work needs to be done to improve the use of this new cell-permeable spin probe. Relating to TBAR production, no differences were observed between groups. Siveski-Iliskovic *et al.* [34] reported an increase in cardiac TBARs after a cumulative dose of doxorubicin of 15 mg/kg administered over a period of 2 weeks, but we must underline the fact that many molecules with no oxidative power can react with TBA to produce TBARs, and this may have distorted our results. No differences in superoxide production between groups were noted when DHE was used in a histological approach. The quenching effect may explain the downward trend in ethidine production due to doxorubicin. The doxorubicin that accumulated in cardiac tissue may have affected emission wavelength and so decreased the fluorescence due to ethidine. Even though no increase in oxidative stress in tissue was noted, immunohistochemistry revealed a greater degree of cardiac inflammation in the D group, suggesting that anthracycline is cardiotoxic. This inflammatory myocardial state due to doxorubicin was not prevented by perindopril. This finding is in accordance with the work of Shibakura *et al.* [42] who reported that doxorubicin induced MCP-1 production in small-cell lung carcinoma cells.

The inflammatory process associated with oxidative stress-induced tissue injury is known to induce dysfunction of the kallikrein-kinin system. [43] It was thus interesting to study the constitutive B_2 receptor and the inducible B_1 receptor in this model of anthracycline cardiotoxicity. Furthermore, it is important to underline the fact that the beneficial effect of ACEI in heart failure seems to involve regulation of kinin receptors. [44] In our study, B_1 receptor mRNA and binding sites were not significantly increased in the myocardium 7 days after the final injection of doxorubicin. Thus the induction of B_1 receptors by inflammatory

cytokines and oxidative stress [10, 43, 45] which involves MAP-kinase and the nuclear transcription factor NF-κB [46] does not appear to occur in the model of cardiotoxicity induced by doxorubicin. However, B_1 receptor binding sites were increased under perindopril treatment. This is consistent with previous studies which have provided evidence for an up-regulation of B_1 receptors under ACEI therapy. [27, 47, 48] B_2 receptor mRNA followed the same trends as those for B_1 receptors. This receptor is also up-regulated by interleukin-1 and tumor necrosis factor. [49] Thus, the inflammatory state of the heart and the production of cytokines induced by doxorubicin may partly explain this result. Similarly to the effect on B_1 receptors, perindopril induced an upward trend in B_2 receptor expression. This is in accordance with earlier work showing an increase in the number of cell-surface B_2 receptors induced by ACEI treatment. [50] In our study, this increase was significant in the D+P group and could be explained by a possible additive effect of doxorubicin and perindopril treatments on B_2 receptor expression. Further pharmacological studies will be needed to determine whether kinin B_1 and B_2 receptors are cardioprotective or not in this model of cardiomyopathy.

To conclude, i.p. administration of doxorubicin to a total cumulative dose of 10 mg/kg over a period of 10 days induced cardiac dysfunction without heart failure in our rat model. Plasma oxidative stress increased whereas oxidative stress in heart tissue remained stable. Myocardial inflammatory and oxidative stress levels in doxorubicin treated rats were probably too low to increase B_1 and B_2 receptors expression significantly. During treatment with doxorubicin, the use of an ACEI did not prevent cardiac dilation but reduced plasma oxidative stress. One possible limitation of our study is the early evaluation of cardiac remodeling. Hence, complete understanding of anthracycline cardiotoxicity and of the potential beneficial effects of ACEIs will require studies with longer period of treatments.

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REFERENCES

1. Delemasure S, Vergely C, Zeller M, et al. [Preventing the cardiotoxic effects of anthracyclins. From basic concepts to clinical data]. *Ann Cardiol Angeiol (Paris).* 2006;55:104-112.
2. Singal PK, Iliskovic N. Doxorubicin-induced cardiomyopathy. *N Engl J Med.* 1998;339:900-905.
3. Horenstein MS, Vander Heide RS, L'Ecuyer TJ. Molecular basis of anthracyclineinduced cardiotoxicity and its prevention. *Mol Genet Metab.* 2000;71:436-444.
4. Delemasure S, Sicard P, Lauzier B, et al. Acute administration of epirubicin induces myocardial depression in isolated rat heart and production of radical species evaluated by electron spin resonance spectroscopy. *J Cardiovasc Pharmacol.* 2007;50:647-653.
5. Ueno M, Kakinuma Y, Yuhki K, et al. Doxorubicin induces apoptosis by activation of caspase-3 in cultured cardiomyocytes in vitro and rat cardiac ventricles in vivo. *J Pharmacol Sci.* 2006;101:151-158.
6. Wang S, Konorev EA, Kotamraju S, et al. Doxorubicin induces apoptosis in normal and tumor cells via distinctly different mechanisms. intermediacy of H_2O_2- and p53-dependent pathways. *J Biol Chem.* 2004;279:25535-25543.
7. Huber SA. Doxorubicin-induced alterations in cultured myocardial cells stimulate cytolytic T lymphocyte responses. *Am J Pathol.* 1990;137:449-456.
8. Ito H, Miller SC, Billingham ME, et al. 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.
9. Minotti G, Recalcati S, Mordente A, et al. The secondary alcohol metabolite of doxorubicin irreversibly inactivates aconitase/iron regulatory protein-1 in cytosolic fractions from human myocardium. *Faseb J.* 1998;12:541-552.
10. Couture R, Harrisson M, Vianna RM, et al. Kinin receptors in pain and inflammation. *Eur J Pharmacol.* 2001;429:161-176.
11. Cheng CP, Onishi K, Ohte N, et al. Functional effects of endogenous bradykinin in congestive heart failure. *J Am Coll Cardiol.* 1998;31:1679-1686.
12. Bast A, Haenen GR, Bruynzeel AM, et al. Protection by flavonoids against anthracycline cardiotoxicity: from chemistry to clinical trials. *Cardiovasc Toxicol.* 2007;7:154-159.
13. Berthiaume JM, Oliveira PJ, Fariss MW, et al. Dietary vitamin E decreases doxorubicin-induced oxidative stress without preventing mitochondrial dysfunction. *Cardiovasc Toxicol.* 2005;5:257-267.
14. Bjelogrlic SK, Radic J, Jovic V, et al. Activity of d,l-alpha-tocopherol (vitamin E) against cardiotoxicity induced by doxorubicin and doxorubicin with cyclophosphamide in mice. *Basic Clin Pharmacol Toxicol.* 2005;97:311-319.
15. Danesi F, Malaguti M, Nunzio MD, et al. Counteraction of adriamycin-induced oxidative damage in rat heart by selenium dietary supplementation. *J Agric Food Chem.* 2006;54:1203-1208.
16. El-Demerdash E, Ali AA, Sayed-Ahmed MM, et al. New aspects in probucol cardioprotection against doxorubicin-induced cardiotoxicity. *Cancer Chemother Pharmacol.* 2003;52:411-416.
17. Wouters KA, Kremer LC, Miller TL, et al. Protecting against anthracycline-induced myocardial damage: a review of the most promising strategies. *Br J Haematol.* 2005;131:561-578.
18. Outomuro D, Grana DR, Azzato F, et al. Adriamycin-induced myocardial toxicity: new solutions for an old problem? *Int J Cardiol.* 2007;117:6-15.

19. Singal PK, Li T, Kumar D, et al. Adriamycin-induced heart failure: mechanism and modulation. *Mol Cell Biochem.* 2000;207:77-86.
20. Effects of enalapril on mortality in severe congestive heart failure. Results of the Cooperative North Scandinavian Enalapril Survival Study (CONSENSUS). The CONSENSUS Trial Study Group. *N Engl J Med.* 1987;316:1429-1435.
21. Cardinale D, Colombo A, Sandri MT, et al. Prevention of high-dose chemotherapyinduced cardiotoxicity in high-risk patients by angiotensin-converting enzyme inhibition. *Circulation.* 2006;114:2474-2481.
22. Dudley SC, Jr., Hoch NE, McCann LA, et al. Atrial fibrillation increases production of superoxide by the left atrium and left atrial appendage: role of the NADPH and xanthine oxidases. *Circulation.* 2005;112:1266-1273.
23. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem.* 1976;72:248-254.
24. Wada R, Tifft CJ, Proia RL. Microglial activation precedes acute neurodegeneration in Sandhoff disease and is suppressed by bone marrow transplantation. *Proc Natl Acad Sci U S A.* 2000;97:10954-10959.
25. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods.* 2001;25:402-408.
26. Hunter WM, Greenwood FC. Preparation of iodine-131 labelled human growth hormone of high specific activity. *Nature.* 1962;194:495-496.
27. Ongali B, Buck Hde S, Cloutier F, et al. Chronic effects of angiotensin-converting enzyme inhibition on kinin receptor binding sites in the rat spinal cord. *Am J Physiol Heart Circ Physiol.* 2003;284:H1949-1958.
28. Cloutier F, de Sousa Buck H, Ongali B, et al. Pharmacologic and autoradiographic evidence for an up-regulation of kinin B(2) receptors in the spinal cord of spontaneously hypertensive rats. *Br J Pharmacol.* 2002;135:1641-1654.
29. Regoli D, Nsa Allogho S, Rizzi A, et al. Bradykinin receptors and their antagonists. *Eur J Pharmacol.* 1998;348:1-10.
30. Hock FJ, Wirth K, Albus U, et al. Hoe 140 a new potent and long acting bradykininantagonist: in vitro studies. *Br J Pharmacol.* 1991;102:769-773.
31. Nazarali AJ, Gutkind JS, Saavedra JM. Calibration of 125I-polymer standards with 125I-brain paste standards for use in quantitative receptor autoradiography. *J Neurosci Methods.* 1989;30:247-253.
32. Okumura K, Jin D, Takai S, et al. Beneficial effects of angiotensin-converting enzyme inhibition in adriamycin-induced cardiomyopathy in hamsters. *Jpn J Pharmacol.* 2002;88:183-188.
33. Ishimitsu T, Honda T, Ohta S, et al. Cardiorenal protective effects of year-long antihypertensive therapy with an angiotensin-converting enzyme inhibitor or a calcium channel blocker in spontaneously hypertensive rats. *Am J Hypertens.* 2006;19:1233-1240.
34. 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.
35. Bertani T, Rocchi G, Sacchi G, et al. Adriamycin-induced glomerulosclerosis in the rat. *Am J Kidney Dis.* 1986;7:12-19.
36. Rabelo E, De Angelis K, Bock P, et al. Baroreflex sensitivity and oxidative stress in adriamycin-induced heart failure. *Hypertension.* 2001;38:576-580.
37. Bertinchant JP, Polge A, Juan JM, et al. Evaluation of cardiac troponin I and T levels as markers of myocardial damage in doxorubicin-induced cardiomyopathy rats, and their relationship with echocardiographic and histological findings. *Clin Chim Acta.* 2003;329:39-51.

38. Tokudome T, Mizushige K, Noma T, et al. Prevention of doxorubicin (adriamycin)-induced cardiomyopathy by simultaneous administration of angiotensin-converting enzyme inhibitor assessed by acoustic densitometry. *J Cardiovasc Pharmacol.* 2000;36:361-368.
39. Kohyama J, Abe H, Shimazaki T, et al. Brain from bone: efficient "metadifferentiation" of marrow stroma-derived mature osteoblasts to neurons with Noggin or a demethylating agent. *Differentiation.* 2001;68:235-244.
40. Chen S, Garami M, Gardner DG. Doxorubicin selectively inhibits brain versus atrial natriuretic peptide gene expression in cultured neonatal rat myocytes. *Hypertension.* 1999;34:1223-1231.
41. Erhola M, Kellokumpu-Lehtinen P, Metsa-Ketela T, et al. Effects of anthracyclinbased chemotherapy on total plasma antioxidant capacity in small cell lung cancer patients. *Free Radic Biol Med.* 1996;21:383-390.
42. Shibakura M, Niiya K, Kiguchi T, et al. Induction of IL-8 and monocyte chemoattractant protein-1 by doxorubicin in human small cell lung carcinoma cells. *Int J Cancer.* 2003;103:380-386.
43. Couture R, Girolami JP. Putative roles of kinin receptors in the therapeutic effects of angiotensin 1-converting enzyme inhibitors in diabetes mellitus. *Eur J Pharmacol.* 2004;500:467-485.
44. Witherow FN, Helmy A, Webb DJ, et al. Bradykinin contributes to the vasodilator effects of chronic angiotensin-converting enzyme inhibition in patients with heart failure. *Circulation.* 2001;104:2177-2181.
45. Marceau F, Hess JF, Bachvarov DR. The B1 receptors for kinins. *Pharmacol Rev.* 1998;50:357-386.
46. Campos MM, Souza GE, Calixto JB. In vivo B1 kinin-receptor upregulation. Evidence for involvement of protein kinases and nuclear factor kappaB pathways. *Br J Pharmacol.* 1999;127:1851-1859.
47. Marin-Castano ME, Schanstra JP, Neau E, et al. Induction of functional bradykinin b(1)-receptors in normotensive rats and mice under chronic Angiotensin-converting enzyme inhibitor treatment. *Circulation.* 2002;105:627-632.
48. Duguay D, Der Sarkissian S, Kouz R, et al. Kinin B2 receptor is not involved in enalapril-induced apoptosis and regression of hypertrophy in spontaneously hypertensive rat aorta: possible role of B1 receptor. *Br J Pharmacol.* 2004;141:728-736.
49. Yayama K, Nagaoka M, Takano M, et al. Expression of kininogen, kallikrein and kinin receptor genes by rat cardiomyocytes. *Biochim Biophys Acta.* 2000;1495:69-77.
50. Sato T, Inagaki A, Uchida K, et al. Diabetes mellitus after transplant: relationship to pretransplant glucose metabolism and tacrolimus or cyclosporine A-based therapy. *Transplantation.* 2003;76:1320-1326.

FIGURE LEGENDS:

FIGURE 1: Functional parameters in control group, doxorubicin (D) group (1 mg/kg/day, 10 days), perindopril (P) group (2 mg/kg/day, 17 days) and D+P group treated with doxorubicin and perindopril at the same dose, during the protocol. (A) Body weight evolution (* $p < 0.05$ D and D+P vs Control; † $p < 0.05$ P vs D and D+P). (B) Heart weight to body weight ratio at day 18 († $p < 0.05$ Control vs P; * $p < 0.05$ D vs D+P).

FIGURE 2: Systolic blood pressure (SBP) evolution in control group, doxorubicin (D) group (1 mg/kg/day, 10 days), perindopril (P) group (2 mg/kg/day, 17 days) and D+P group treated with doxorubicin and perindopril at the same dose, during the protocol. (* $p < 0.05$ at day 6 and 9 vs day 0 in D group; † $p < 0.05$ from day 6 to day 17 vs day 0 in P group).

FIGURE 3: Left ventricular end-diastolic diameter (LVDd) in control group, doxorubicin (D) group (1 mg/kg/day, 10 days), perindopril (P) group (2 mg/kg/day, 17days) and D+P group treated with doxorubicin and perindopril at the same dose, at day 18 (* $p < 0.05$ at day 0 vs day 18 in D group; † $p < 0.05$ at day 0 vs day 18 in D+P group).

FIGURE 4: MCP-1 localization (red color) by immunohistochemistry in control myocardium (A) and in doxorubicin-treated group myocardium (B).

FIGURE 5: B_1 and B_2 kinin receptors in myocardium, analyzed by RT-PCR (A) and autoradiography (B) at day 18, in control group, doxorubicin (D) group (1 mg/kg/day, 10 days), perindopril (P) group (2 mg/kg/day, 17 days) and D+P group (*$p<0.05$ vs control).