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Prominent cardioprotective effects of third generation beta blocker nebivolol against anthracycline-induced cardiotoxicity using the model of isolated perfused rat heart

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ABSTRACT

Nebivolol is a cardioselective beta-blocker (BB) currently used for the treatment of hypertension. It has mild vasodilating properties attributed to its interaction with the L-arginine/nitric oxide pathway, a property not shared by other BBs. Carvedilol is a nonselective ß-adrenergic receptor antagonist that also blocks α1-adrenergic receptors and is a potent antioxidant. Anthracyclines (ANTs), daunorubicin and doxorubicin, are commonly used in the treatment of several tumours, but their cardiac toxicity prevents their use at maximum myelotoxic doses, representing an important problem. In this study, we have evaluated the role of these BBs administered in combination with ANTs (daunorubicin and doxorubicin) on a reduction in cardiac toxicity. The combination of BB and ANTs has reduced the release of GSSG and GSH; in particular, co-treatment with nebivolol to ANTs has shown a significant reduction. The total integrated creatine kinase and troponin T activities were improved by BB and ANTs co-treatment. A significant reduction of their release was observed when hearts were treated with nebivolol. Cardiac tissue activity of gluthatione reductase was not significant and similar among experimental groups. In contrast, gluthatione peroxidise, Mn-superoxide dismutase and nitrite/nitrate release were increased after co-treatment with nebivolol. Finally, three parameters have been used to evaluate the cardiac toxicity of ANTs: the left ventricular pressure developed under a constant perfusion pressure (LVDP), the rate of variation of this parameter during systole (contractility) $(LV/dt)_{max}$ and during diastole (relaxation) $(LV(dP/dt)_{min}$. Combination with BB has shown a reduction in cardiac toxicity; in particular, nebivolol has exerted the most significant cardioprotective effect.

1. Introduction

The common use of anthracyclines in various malignancies is hindered by their cardiac toxicity, which remains a major problem 50 years after the discovery of daunorubicin and doxorubicin and their introduction in clinics.[1] This toxicity has elicited a large number of studies aimed both at understanding the mechanisms involved in the cardiac toxicity of

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these drugs and at circumventing it. Several approaches have been considered in this respect: (1) the development of anthracycline analogues devoid of cardiac toxicity; (2) the use of alternative schedules of administration such as protracted infusions; (3) the encapsulation of the anticancer drug in liposomal or other particulate formulations; (4) the combination of anthracycline with a cardioprotector. In this last area, a large number of potential drugs have been developed in preclinical models, such as free radical scavengers and anti-oxidants. However, only one drug has proven its efficacy in the clinical setting: dexrazoxane (ICRF187), a prodrug of an intracellular iron chelator (ADR925) which decreases rate of the Fenton reaction, a pathway leading to doxorubicin-induced free radical formation.[2]

Similarly, the β-blocker (BB) carvedilol seems to prevent the development of cardiac toxicity induced by doxorubicin in rats[3] and in patients receiving anthracyclines.[4] We have already shown that the isolated perfused rat heart may represent a useful model for the preclinical evaluation of cardiac performance and interventions.[5,6] The short term model of isolated perfused rat heart is also the best model to evaluate anthracycline cardiotoxicity and its circumvention. [7–9] These preclinical evidences justify the use of this short-term model to predict the cardiac side effects of anthracyclines in various conditions.

The third generation BB nebivolol exhibited a potent cardioprotection through the nitric oxide pathway.[10–13] Since there is the scientific rationale to test further cardioprotective effects of nebivolol, we have evaluated in this research the role of this compound (alone and in comparison to the other BB carvedilol) administered in combination with anthracyclines (daunorubicin and doxorubicin) currently used in the clinical setting.

2. Methods

2.1. Drugs and chemicals

Doxorubicin and daunorubicin (Sigma) were diluted with sterile water to a final concentration of 5 mg/ml and stored at +4 °C for a maximum of 4 days. Carvedilol and nebivolol (Sigma) were diluted with sterile water at a final concentration of 20 mg/ml and kept at +4 °C for a maximum of 4 days. In pilot studies, we evaluated the best doses of drugs exerting cardioprotective effects.

2.2. Experimental animals

All experiments reported here were done in accordance with the guidelines of the National Institutes of Heath (NIH) and Ministero della salute (Italy). Treatments were administered i.p. to male Sprague Dawley rats aged 10–12 weeks every other day for 12 days. The rats were weighed every 2 days and assessed for possible abnormalities (ascites, bleeding, diarrhoea, etc.). Rats were killed on the 12th day after the first injection; hearts were removed and perfused, and cardiac functional parameters were monitored as described below.

Treatment included an anthracycline at a selected dose providing, whenever possible, equi-cardiotoxicity. Doxorubicin was used at 3 mg/kg per day (18 mg/kg total dose), daunorubicin at 4 mg/kg per day (24 mg/kg total dose). These doses were chosen after multiple trials and corresponded to those giving an acceptable general toxicity together with major cardiac functional symptoms. The purpose of the study was not to compare the two anthracyclines, but to evaluate the improvement of cardiac symptoms when combined with BBs (the NO-releasing beta-blocker nebivolol or the beta-blocker with antioxidant properties carvedilol). For each anthracycline studied, groups of rats were treated (1) with the anthracycline alone at the selected dose, (2) with the anthracycline combined with BBs or (3) with shadow injections of saline.

2.3. Perfusion of isolated rat hearts

Rats were heparinised i.p. (5000 U/kg) and anaesthetised with diethylether. The heart was quickly excised and soaked in Krebs-Henseleit solution at +4 °C. Coronary perfusion was initiated through a short cannula in the aortic root and maintained at a constant pressure of 90 mm Hg in a non-recirculating way by the Langendorff technique as described in detail.[6] Perfusion pressure was measured by a P23Db transducer (Bentley Trantec) connected to the aortic infusion cannula. The heart was electrically paced at a rate of 300 beats/min (5 Hz) through stimulator-activated stainless steel electrodes placed on the heart. A latex balloon attached to one end of a polyethylene catheter was placed in the left ventricle through the mitral valve. The catheter was filled with water and the other end linked to an electronic amplifier (Thomson Medical) via a second P23Db transducer.

The coronary perfusion pressure and the left ventricular pressure were recorded on a computer allowing continuous monitoring of heart rate, left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure (LVEDP), left ventricular developed pressure (LVDP = LVSP-LVEDP) and the maximal and minimal first derivatives of LVDP as a function of time [$LV(dp/dt)_{max}$ and $LV(dp/dt)_{min}$], respectively. The perfusate consisted of a modified Krebs–Henseleit buffer, pH 7.4, containing NaCl (118 mM), KCl (4.7 mM), $MgSO_4$ (1.2 mM), KH_2PO_4 (1.2 mM), $NaHCO_3$ (25 mM), glucose (11 mM), $CaCl_2$ (0.95 mM) and insulin (10 i.u./l). It was continuously bubbled with a mixture of 95% O_2/5% CO_2 and maintained at 37 °C. The latex balloon inserted in the left ventricle was periodically dilated with distilled water in order to produce a LVEDP of 5–6 mm Hg. After 30–45 min stabilisation, necessary to reach the maximal functional cardiac values, the above parameters were recorded.

2.4. Evaluation of oxidative stress, cardiac damage and nitrite/nitrate levels

At various time-selected points (the supernatant was used for the determination of glutathione (GSSG) and reduced glutathione (GSH) release), additional 0.4-ml aliquots of coronary effluent were simultaneously drawn into a syringe containing 100 μl of 10 mM EDTA and 50 mM *N*-ethyl-maleimide in 100 mM K-phosphate buffer at pH 7.4.[5] Concentrations of total glutathione (i.e. GSSG + GSH) and GSH were measured by the glutathione reductase-5,5′- dithiobis-2-nitrobenzoic acid recirculating assay[5] (expressed as nanomoles of GSH equivalents released per minute per gram wet weight). Total inte-

grated creatine kinase (CK) activity over reperfusion was evaluated as described.[5] Troponin T was measured with a commercial immunoassay kit following manufacturer's instructions (Roche Diagnostics, Indianapolis, USA). Tissue glutathione reductase and peroxidase and Mn-superoxide dismutase were determined spectrophotometrically as described.[5] The NO radical has a short half-life, so the plasma concentrations of NO stable end-products, nitrite/nitrate, were used as an indicator of vascular NO production. Combined plasma nitrite/nitrate concentrations were measured using a commercial colorimetric kit (Calbiochem), according to the established method based on the use of Griess reagent, as described previously.[14]

2.5. *Statistical analysis*

Statistical comparisons between untreated and treated groups were made by Student's *t*-test after ANOVA assumption of the validity of *t*-test; all data are expressed as mean value ± S.D. Statistical significance was determined as a *p*-value below 0.05.

3. Results

Anthracyclines exerted a marked general toxicity at the doses used in these experiments. Diarrhoea remained relatively infrequent but spontaneous bleeding occurred in around 30% of the animals. A weight loss of around 10% of initial weight was present for both anthracyclines on the 12th day, to be compared to the 20% weight gain recorded in control animals during the same period (Table 1).

Oxidative stress pathways were improved significantly by BB production (Table 2). Similarly, GSSG and GSH release were improved by such treatments (Table 2). The combination of beta-blockers and anthracyclines reduced the release of GSSG and GSH; in particular, co-treatment with nebivolol to anthracyclines showed a significant reduction. The total integrated creatine kinase (CK) and Troponin T activities (a recognised marker of cardiac damage) were improved by beta-blockers and anthracyclines co-treatment (Table 2). A significant reduction of this release was observed when hearts were treated with nebivolol. Cardiac tissue activity of gluthatione reductase was similar among experimental groups. In contrast, gluthatione

Table 1 – General toxicity of the anthracyclines

	Early death	Diarrhoea	Bleeding	Weight variation (%)	*p*-Value *versus* control
Control	0/12	0/12	0/12	+21.0 ± 4.6	
Doxorubicin 3 mg/kg	0/18	0/18	6/18	−10.0 ± 6.5	2×10^{-10}
Daunorubicin 4 mg/kg	3/21	7/21	8/21	−20.9 ± 9.7	4×10^{-12}

The doses indicated in the first column are daily doses repeated six times over 12 days. Results are given as means ± S.D. *p*-Values have been calculated using Student's *t*-test; values were compared versus control.

Table 2 – Values of GSH, GR, GSSG, CK, GP and Mn-SD in experimental groups

	GSH (nmoles/GSH equivalents/g wet weight)	GR (mU/mg prot)	GSSG (nmoles/GSH equivalents/g wet weight)	CK (I.U./g/dry weight)	Troponin T (mg/L)	GP (mU/mg prot) Mn-SD (units/mg prot)	
Control	48 ± 8	2.5 ± 0.2	300 ± 15	659.8 ± 61.9	2.5 ± 0.9	60 ± 8	2.0 ± 0.4
Daunorubicin	77 ± 11	2.6 ± 0.3	560 ± 56	847.6 ± 53.4	3.9 ± 1.4	56 ± 7	1.9 ± 0.3
Daunorubicin + nebivolol	60 ± 8*	2.4 ± 0.3	400 ± 26	622 ± 62*	2.8 ± 0.6*	69 ± 5*	2.3 ± 0.4*
Daunorubicin + carvedilol	67 ± 13	2.5 ± 0.3	476 ± 33	689 ± 72	3.4 ± 0.9	63 ± 8	2.1 ± 0.4
Doxorubicin	80 ± 13	2.4 ± 0.4	595 ± 67	923 ± 89	4.3 ± 1.2	55 ± 9	1.8 ± 0.3
Doxorubicin + nebivolol	66 ± 9*	2.5 ± 0.3	420 ± 31*	700 ± 47*	3.2 ± 0.8*	67 ± 8*	2.3 ± 0.2*
Doxorubicin + carvedilol	73 ± 12	2.5 ± 0.4	522 ± 28	803 ± 73	3.9 ± 1.3	63 ± 9	2.1 ± 0.2

Results are given as means ± SD. *p*-Values have been calculated using Student's *t*-test; values were compared versus control.

Table 3 – Concentration of nitrite/nitrate among groups

	Control	Daunorubicin	Dau + Nebi	Dau + Carv	Doxorubicin	Doxo + Nebi	Doxo + Carv
Nitrite/nitrate (μM)	15.8 ± 1.9	6.4 ± 1.1[a]	13.8 ± 1.9[b]	10.4 ± 1.1[b]	5.5 ± 1.8[a]	12.4 ± 1.2[b]	9.6 ± 1.7[b]

Data are means ± S.E.M. of five animals per group.
a $P < 0.001$ versus vehicle-treated rats.
b $P < 0.01$ versus respective Dau or Doxo by ANOVA.

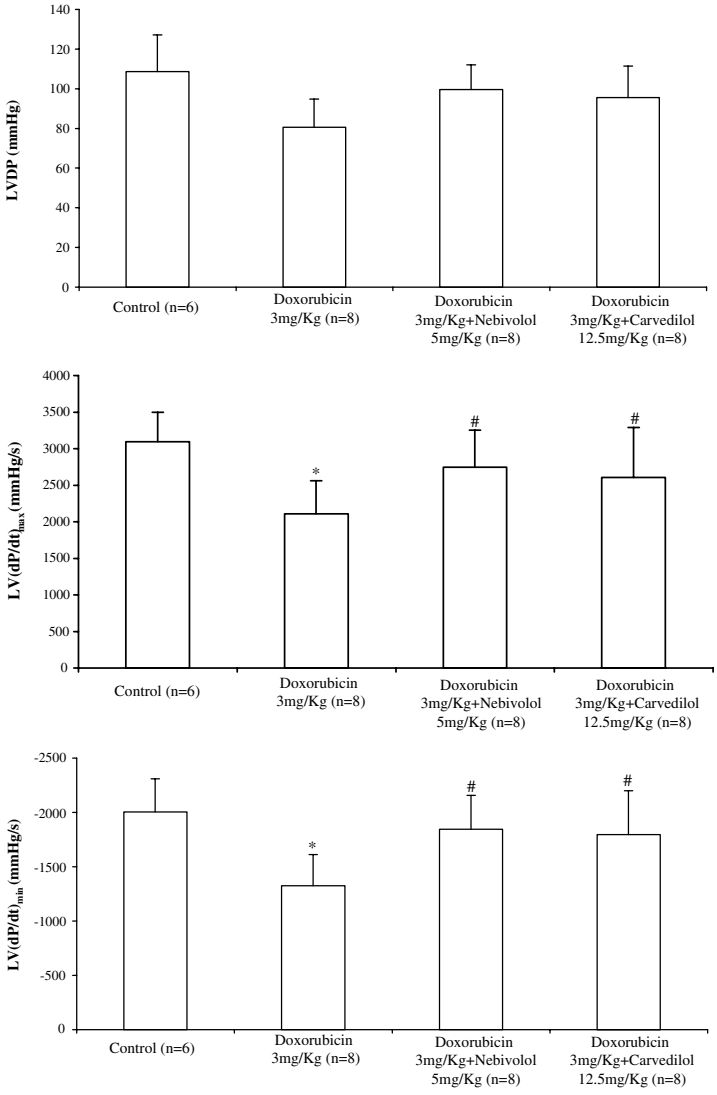

Fig. 1 – Doxorubicin treatment effects on cardiac toxicity: Effects of doxorubicin (3 mg/Kg) alone and in combination with nebivolol (5 mg/Kg) and carvedilol (12.5 mg/Kg) on LVDP, $LV(dP/dt)_{max}$, and $LV(dP/dt)_{min}$, evaluated with the model of isolated perfused rat heart. Results are given as means ± S.D. *$p < 0.05$ were compared *versus* control by ANOVA; #$p < 0.05$ were compared *versus* Doxorubicin/Daunorobicin by ANOVA.

peroxidase and Mn-superoxide dismutase were increased after co-treatment with nebivolol and anthracyclines (Table 2). Finally, nitric oxide release was depressed by both anthracyclines and restored by beta-blockers indicating the protective action of the drug on endothelium (Table 3). This effect on nitric oxide was more pronounced when nebivolol was used.

Three functional parameters were used to evaluate the cardiac toxicity of anthracyclines and drug–anthracycline combinations: the left ventricular pressure developed under a constant perfusion pressure (LVDP), the rate of variation of this parameter during systole (contractility) $(LV/dt)_{max}$ and during diastole (relaxation) $(LV(dP/dt)_{min}$ (Figs. 1 and 2). In our experimental conditions, both anthracyclines exerted a significant cardiac toxicity, which was generally more pronounced on relaxation than on the other parameters. When combined with drugs, there was a reduction in cardiac toxicity. Nebivolol exerted the most significant cardioprotective effects (Fig. 1a–c; Fig. 2a–c).

Overall, GSSG levels inversely correlated with the recovery of LV(dP/dt)max nebivolol after doxorubicin treatment ($r = -0.65$, $P < 0.01$). Moreover, the increase of nitrite/nitrate levels was positively correlated with LV(dP/dt)max with nebivolol after doxorubicin treatment ($r = 0.47$; $P < 0.05$).

4. Discussion

The present study shows a prominent cardioprotection elicited by nebivolol, and, to a lesser degree, carvedilol, in a rat model of anthracyclines-mediated toxicity.

Anthracyclines have gained widespread use in the treatment of haematological malignancies and solid tumours, but their cumulative toxicity on the myocardium prevents

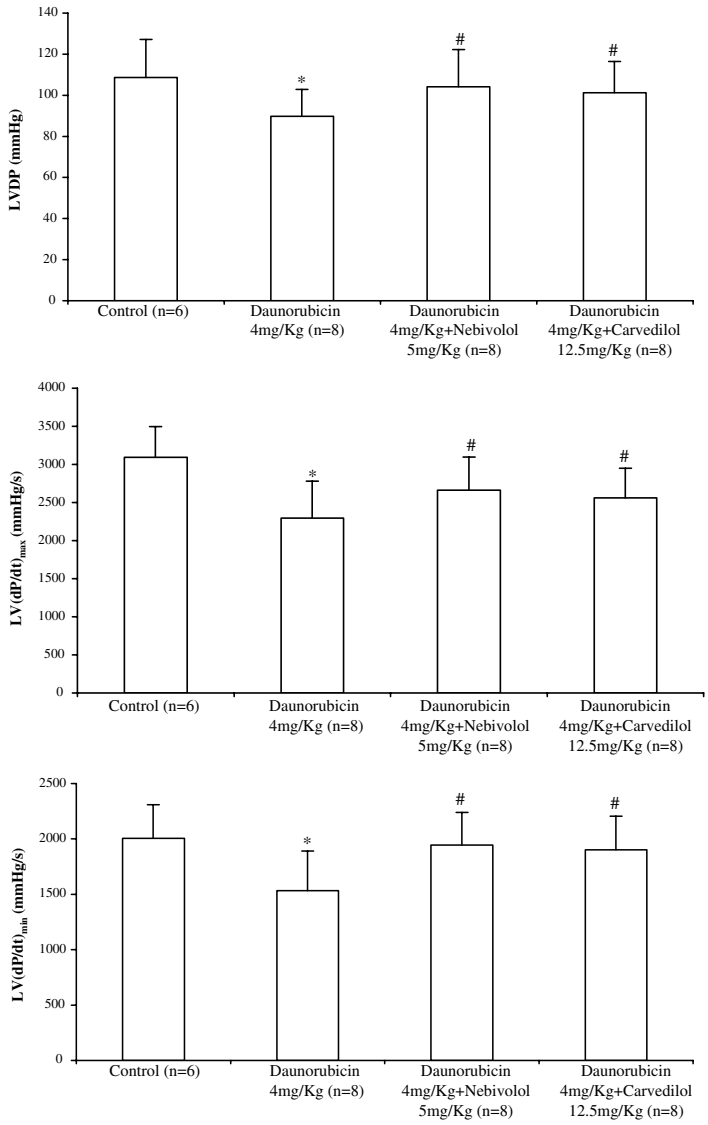

Fig. 2 – Daunorobicin treatment effects on cardiac toxicity: Effects of daunorubicin (4 mg/Kg) alone and in combination with nebivolol (5 mg/Kg) and carvedilol (12.5 mg/Kg) on LVDP, $LV(dP/dt)_{max}$, and $LV(dP/dt)_{min}$, evaluated with the model of isolated perfused rat heart. Results are given as means ± S.D. *p <0.05 were compared *versus* control by ANOVA; #p < 0.05 were compared *versus* Doxorubicin/Daunorobicin by ANOVA.

their use at their maximum myelotoxic doses during the optimal number of therapeutic cycles required.[9] Heart damage due to anthracycline therapy is a considerable and serious problem. It reduces the quality of life and can even cause premature death. Also, when heart damage occurs during therapy the maximum cumulative dose of anthracyclines needs to be limited and as a result the efficacy of anthracycline therapy may be reduced. Therefore, it is extremely important to identify methods to reduce or even prevent anthracycline-induced cardiotoxicity.

Our research provides important evidence on preventing anthracycline-induced cardiotoxicity with regard to the use of different BBs, and the use of two different anthracyclines in a rat model. This model of isolated perfused rat heart appears as useful for a rapid preclinical evaluation of anthracycline cardiotoxicity. As a model, it does not pretend to mimic the delayed cardiac toxicity of anthracyclines in humans, which generally occurs 1–2 years after treatment completion in adults, and even much later in children.[15] In clinics, the myocardial cellular lesions induced by anthracyclines occur rapidly after each administration, as evidenced from the early elevation of serum troponin levels,[16] but there is, to a certain degree, a physiological compensation to the loss of cardiac fibres, explaining the delay between anthracycline administration and the occurrence of heart failure. However, it appears possible to detect functional alterations shortly after administration and to compare, in a short-term model such as the one we have implemented, the ability of different drugs and drug combinations to induce heart fibre damage. This model is useful to predict anthracycline cardiotoxicity in various situations.[9] Consistently, in the present study, beta blockers reduced troponin levels after exposure to anthracyclines.

We show in this report that nitric oxide (NO)-releasing beta blocker [10] exert a significant protective effect on the cardiac toxicity induced by anthracyclines currently used in the clinical setting, in part by a restoring of NO levels. Indeed, BBs have been used to treat ischemic heart disease, due to negative chronotropic and inotropic properties, thus inducing a decrease in myocardial consumption of oxygen and nutrients, allowing a better balance between nutritional needs and the supply provided by the coronary blood flow. Recent developments in cell biology allow us to understand that not all BBs are equal, as their intracellular mechanisms of action can be very different.[17] There is no clinical trial with a large number of patients evaluating the efficacy of BBs in anthracycline-induced cardiomyopathy (ACM), since it may be difficult to carry out such a study for this rare form of heart failure.[18] In this research we have evaluated the role of these compounds (nebivolol and carvedilol), administered in combination with anthracyclines (daunorubicin and doxorubicin), on a reduction in cardiac toxicity. Nebivolol is a cardioselective BB currently used for the treatment of hypertension.[19] It has mild vasodilating properties attributed to its interaction with the L-arginine/NO pathway, a property not shared by other beta blockers. Carvedilol is a nonselective β-adrenergic receptor antagonist that also blocks α1-adrenergic receptors and is a potent antioxidant.[20]

Our results show a beneficial effect of BBs on anthracycline treated hearts. In particular, the use of nebivolol or carvedilol with anthracyclines have reduced the release of GSSG and GSH. Since the most important property of carvedilol is antioxidative profile and nebivolol predominantly effects NO pathway, the common protective pathway of nebivolol and carvedilol could be the intrinsinc beta blocker properties coupled to antioxidant and NO release. Cardiac tissue activity of gluthatione reductase is not significant and similar among experimental groups. In contrast, gluthatione peroxidase and Mn-superoxide dismutase were increased. Three cardiac parameters have been used to evaluate the cardiac toxicity of anthracyclines and drug–anthracycline combinations: the left ventricular pressure developed under a constant perfusion pressure (LVDP), the rate of variation of this parameter during systole (contractility) $(LV/dt)_{max}$ and during diastole (relaxation) $(LV(dP/dt)_{min}$. This combination has shown a reduction in cardiac toxicity; in particular, nebivolol has exerted the most significant cardioprotective effects. The restoring of LV/dt_{max} was correlated to reduced GSSG and increased NO levels. This property is consistent with experimental and clinical data indicating a plethora of beneficial experimental and clinical cardioprotective effects of nebivolol via the NO pathway (reviewed in Refs[21,22]) which also suggests its possible clinical use against cardiac toxicity induced by anthracyclines. Finally, acute left ventricular decompensation in patients following anthracycline exposure may not be solely attributed to drug assumption, but viral etiologies should be considered.[23]

Conflict of interest statement

None declared.

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