

## Aging and Cardiac Function

# Unchanged G-Protein-Coupled Receptor Kinase Activity in the Aging Human Heart

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<b>OBJECTIVES</b>	We sought to find out whether G-protein-coupled receptor kinase (GRK) activity is also increased in the aging human heart.
<b>BACKGROUND</b>	In the aging and failing human heart, cardiac beta-adrenoceptors (beta-AR) are desensitized. In heart failure (HF), an increase in cardiac GRK activity considerably contributes to this beta-AR desensitization.
<b>METHODS</b>	We assessed GRK activity (by in vitro rhodopsin phosphorylation) in the right atria (RA) from 16 children (mean age $9 \pm 2$ years) and 17 elderly patients (mean age $67 \pm 2$ years) without apparent HF and in the RA from four patients with end-stage HF.
<b>RESULTS</b>	Cytosolic and membranous GRK activities in the RA from children were not significantly different from those in elderly patients; in contrast, cytosolic and membranous GRK activities in the RA from patients with end-stage HF were significantly increased.
<b>CONCLUSIONS</b>	In contrast to the failing human heart, in the aging human heart, GRK activity is not increased. Thus, GRK activity appears to not play an important role in beta-AR desensitization in the aging human heart. (J Am Coll Cardiol 2003;42:1487-92) © 2003 by the American College of Cardiology Foundation

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It is now generally accepted that with aging the functional responsiveness of the human cardiac beta-adrenoceptor (beta-AR) system decreases (1-3). In addition, we have previously shown that not only beta-AR-mediated effects but all effects mediated by cyclic adenosine monophosphate (cAMP) are blunted in the aging human heart (4). Thus, in this respect the aging human heart resembles the failing human heart, since in chronic heart failure (HF) also all effects mediated by cAMP are reduced (2,3). One important mechanism that plays a significant role in cardiac beta-AR desensitization in chronic HF is an increase in activity of G-protein-coupled receptor kinase (GRK) that enzyme that phosphorylates agonist-occupied receptors, and by this uncouples receptors from the effector mechanism (3,5,6). This increase in cardiac GRK activity appears to be due to chronic activation of cardiac beta-ARs by the increased sympathetic nervous system activity (6,7).

It has repeatedly been reported that with aging sympathetic activity also increases (3,8,9). Thus, it might also be true that, in the aging human heart, an increase in GRK activity due to chronic beta-AR stimulation by increased sympathetic activity might contribute to beta-AR desensitization. To test this hypothesis, in this study we assessed GRK activity in the right atria (RA) from patients of different ages without apparent HF.

## METHODS

**Right atrial appendages.** Right atrial appendages were obtained from: 1) 16 children (9 males and 7 females; age 3 months to 21 years [mean  $9 \pm 2$  years]) with acyanotic congenital heart disease who underwent open-heart surgery because of a ventricular septal defect ( $n = 7$ ) or atrial septal defect ( $n = 9$ ). Their parents had given informed, written consent. None of the patients suffered from acute HF or had been treated with sympathomimetics or sympatholytics for at least three weeks before surgery; and 2) 17 adult patients with coronary artery disease (9 males and 8 females; age 52 to 80 years [mean  $67 \pm 2$ ]) undergoing coronary artery bypass grafting without apparent HF (New York Heart Association [NYHA] functional class 0-I:  $n = 3$ ; NYHA class II:  $n = 14$ ); their ejection fraction was  $67 \pm 5\%$ . All patients gave written, informed consent before surgery. None of these patients had been treated with sympathomimetics or sympatholytics for at least six weeks before surgery. However, patients had received nitrates ( $n = 6$ ), calcium antagonists ( $n = 2$ ), diuretics ( $n = 4$ ), hydroxymethylglutaryl coenzyme A reductase inhibitors ( $n = 4$ ), acetyl salicylic acid ( $n = 5$ ), and occasionally digitalis glycosides ( $n = 1$ ), alone or in combination.

Anesthesiologic premedication and surgery were carried out exactly as recently described (4,10). Right atrial appendages were removed after installation of the cardiopulmonary bypass. Immediately after excision, the specimen were quickly frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$  until further use. The study was approved by the local ethics committees.

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**Abbreviations and Acronyms**

AC	=	adenyl cyclase
AR	=	adrenoceptor
ATP	=	adenosine triphosphate
cAMP	=	cyclic adenosine monophosphate
GRK	=	G-protein-coupled receptor kinase
GTP	=	guanosine triphosphate
HF	=	heart failure
NYHA	=	New York Heart Association
RA	=	right atria, right atrium

In addition, right atrial myocardium was obtained from four male patients with end-stage HF (mean age  $51 \pm 4$  years, NYHA class IV) due to ischemic ( $n = 3$ ) or dilated cardiomyopathy ( $n = 1$ ), with an ejection fraction of  $20 \pm 3\%$ , undergoing heart transplantation and from four organ donors (3 males and 1 female; mean age  $30 \pm 12$  years) whose hearts could not be used for transplantation.

Written, informed consent was obtained from a family member; donor histories revealed no signs of HF.

**Determination of GRK activity.** The GRK activity was assessed as recently described, with minor modifications (11). Briefly, frozen tissue was thawed on ice in 5 ml ice-cold lysis buffer (25 mmol/l Tris-HCl, 5 mmol/l EDTA, and 5 mmol/l EGTA containing 40  $\mu\text{g/ml}$  leupeptin, 40  $\mu\text{g/ml}$  benzamidine, and 40  $\mu\text{g/ml}$  phenylmethane sulfonyl fluoride). Fatty and connective tissue were carefully removed, and 20 to 50 mg of the remaining tissue was transferred into 1 ml lysis buffer, minced with scissors, and homogenized with an Ultra-Turrax (Ultra-Turrax T25; Janke & Kunkel IKA Labortechnik; Staufen, Germany) at 19,000 rpm for 60 s on ice.

Before centrifugation for 30 min at 50,000  $g$  and 4°C, 10  $\mu\text{l}$  of a 5 mol/l NaCl solution (final concentration 50 mmol/l) was added, and the homogenate was incubated for 10 min on ice. After centrifugation, the supernatant ("cytosolic GRK fraction") was carefully removed and transferred into new reaction tubes, quickly frozen in liquid nitrogen, and stored at  $-80^\circ\text{C}$  until further use.

To detach membrane-associated GRK, the pellet was re-suspended in 760  $\mu\text{l}$  lysis buffer + 40  $\mu\text{l}$  5 mol/l NaCl solution (final concentration 250 mmol/l) with the Ultra Turrax for 60 s at 9,500 rpm. After 15 min of incubation on ice, the homogenate was re-centrifuged for 30 min at 50,000  $g$  and 4°C. The supernatant ("membranous GRK fraction") was transferred into new reaction tubes, quickly frozen into liquid nitrogen, and stored at  $-80^\circ\text{C}$  until further use. The protein concentration was determined according to Bradford, with bovine gamma-globulin as standard (12).

Twenty micrograms of cytosolic protein and 10  $\mu\text{g}$  of membrane protein were mixed in duplicate in the dark at

4°C with 200 pmol rhodopsin (prepared from bovine retina), 10 mmol/l  $\text{MgCl}_2$ , 100  $\mu\text{mol/l}$  adenosine triphosphate (ATP), 180 pmol beta-gamma-subunits, and gamma- $^{32}\text{P}$ -ATP (20  $\mu\text{Ci} \sim 0.08$  mmol/l) in a total volume of 60  $\mu\text{l}$ . Light-dependent phosphorylation of rhodopsin was initiated by incubating samples under light for 10 min at 30°C. The reaction was terminated by the addition of 250  $\mu\text{l}$  ice-cold lysis buffer. Samples were immediately centrifuged for 10 min at 10,000  $g$  at room temperature; the supernatants were discarded and the pellets re-suspended in 30  $\mu\text{l}$  twice-concentrated Laemmli buffer by vigorous shaking for 15 min at 30°C (13).

The samples were electrophoresed on 10% sodium dodecyl sulfate-polyacrylamide gels, which were stained with Coomassie brilliant blue, according to Reissner et al. (14), and autoradiographed. The rhodopsin bands (35 kd) were cut off the gel, and their radioactivity was determined by Cerenkov counting.

**Determination of adenylyl cyclase (AC) activity.** The AC activity was assessed by a method repeatedly described in detail elsewhere (4,15).

**Statistical evaluations.** The experimental data given in text and figures are expressed as the mean value  $\pm$  SEM of the number of patients. The significance of differences was estimated by the nonpaired two-tailed Student  $t$  test. All statistical calculations were performed with the Prism program (Graph-Pad Software, San Diego, California). A  $p$  value  $< 0.05$  was considered to be significant.

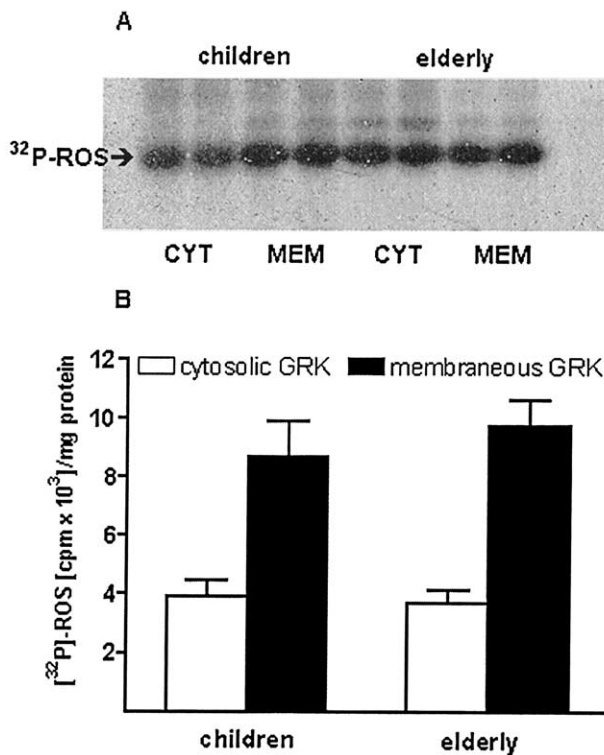
**Drugs used.** Both (alpha- $^{32}\text{P}$ )-ATP and (gamma- $^{32}\text{P}$ )-ATP were purchased from NEN Life Science Products Inc. (Boston, Massachusetts). Purified bovine rhodopsin and the beta-gamma-subunits for the GRK assay were kindly provided by Prof. Dr. M. J. Lohse (University of Würzburg, Germany). All other chemicals were of the highest purity grade commercially available.

## RESULTS

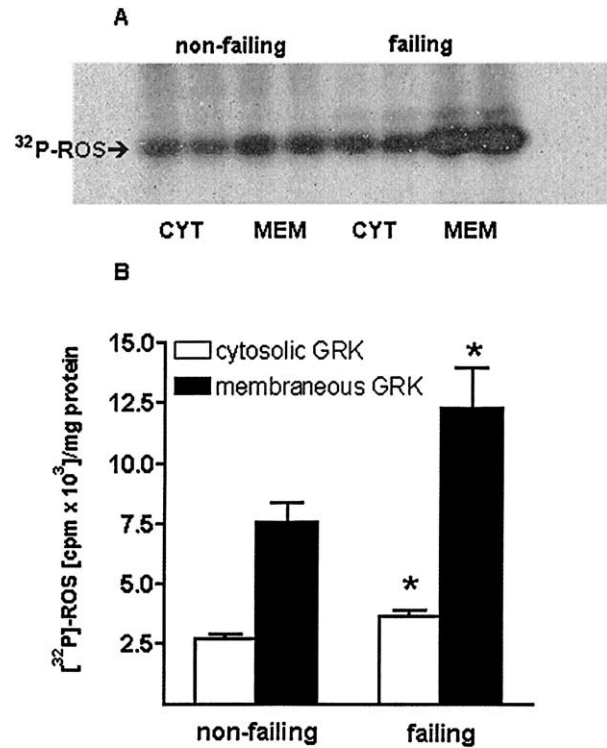
### Right atrial GRK activity in young and elderly patients.

The GRK activity was measured in right atrial cytosol and membranes by assessing their capacity to phosphorylate light-activated rhodopsin. Cytosolic and membranous GRK activities in the RA from children were not significantly different from those in elderly patients. As demonstrated in Figure 1, the GRK pool distribution (cytosolic/membranous GRK activity ratio) was also not significantly different between children ( $31.1 \pm 2\%:68 \pm 2\%$ ) and elderly patients ( $27.5 \pm 1.6\%:72 \pm 1.6\%$ ).

To demonstrate that with our method we are able to detect changes in GRK activity, we next assessed GRK activity in the RA from patients with end-stage HF, as it is well known that in these patients GRK activity is increased (5). Cytosolic as well as membranous GRK activities were significantly higher in failing versus non-failing atria, resulting in a slight but nonsignificant shift in the cytosolic/membranous GRK activity ratio ( $23 \pm 1.6\%:77 \pm 1.6\%$  in



**Figure 1.** (A) Typical autoradiograph of light-dependent in vitro phosphorylation of rhodopsin (<sup>32</sup>P-ROS) by atrial cytosolic (CYT) and membranous (MEM) GRK obtained from a child (3-year-old male; NYHA class 0) and an elderly patient (60-year-old male; NYHA class I). (B) Bar graph showing cytosolic and membranous GRK activity in right atrial appendages obtained from 10 children (5 males and 5 females; mean age 6 ± 2 years [range 3 months to 20 years]; NYHA class 0.9 ± 0.3) and eight elderly patients (4 males and 4 females; mean age 68 ± 3 years [range 59 to 80]; NYHA class 1.9 ± 0.4). Activities were calculated as <sup>32</sup>P incorporated in rhodopsin in cpm × 10<sup>3</sup> per mg protein. Data are given as the mean value ± SEM.



**Figure 2.** (A) Typical autoradiograph of light-dependent in vitro phosphorylation of rhodopsin (<sup>32</sup>P-ROS) by cytosolic (CYT) and membranous (MEM) GRK obtained from the RA from a non-failing (3-year-old male donor; NYHA class 0) and failing (61-year-old male recipient; NYHA class IV) heart. (B) Bar graph showing cytosolic and membranous GRK activity in right atrial appendages obtained from four non-failing (mean age 30 ± 12 years [range 3 to 52]; 3 males and 1 female; NYHA class 0) and four failing (mean age 51 ± 4 years [range 44 to 63]; 4 males; NYHA class IV) hearts. Activities were calculated as <sup>32</sup>P incorporated in rhodopsin in cpm/mg protein. Data are given as the mean value ± SEM. \*p = 0.0381 vs. non-failing heart.

failing vs. 27 ± 1.2%:73 ± 1.2% in non-failing RA, p = 0.092) (Fig. 2).

**Adenylyl cyclase activity in young and elderly patients.**

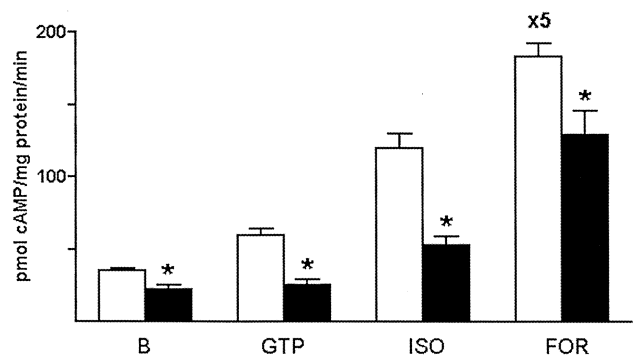
To demonstrate that in our study the right atrial beta-AR-G-protein(s)-AC system differs between children and elderly patients, we finally assessed right atrial AC activity. In agreement with our recently published data (4,15), we found that in atrial membranes from children, basal AC activity, as well as 10 μmol/l guanosine triphosphate (GTP)-, 10 μmol/l isoprenaline-, and 10 μmol/l forskolin-stimulated AC activity, was significantly higher than in atrial membranes from elderly patients (Fig. 3).

**DISCUSSION**

The main finding of this study was that cardiac GRK activity was not different in the RA from children or elderly patients; however, it was significantly increased in the RA from end-stage failing hearts versus non-failing hearts, in agreement with the literature (3,5,6).

**Beta-ARs are desensitized in the aging human heart.** It is well known that with aging, the cardiac beta-AR-G-

protein(s)-AC system declines. We have previously shown that in the RA of patients with an age range very similar (7 days to 83 years) to that in the present study, there was a



**Figure 3.** Adenylyl cyclase activity in right atrial appendages obtained from six children (4 males and 2 females; mean age 13 ± 3 years [range 4 to 21]; open columns) and nine elderly patients (5 males and 4 females; mean age 66 ± 3 years [range 52 to 78]; solid columns). Data are given as the mean value ± SEM. \*p < 0.05 vs. children. B = basal; GTP = 10 μmol/l guanosine triphosphate; ISO = 10 μmol/l isoprenaline; FOR = 10 μmol/l forskolin.

significantly negative correlation between the age of the patients and AC activity. This held true not only for beta-AR-activated AC but also for GTP-, histamine- (via H<sub>2</sub> receptors), serotonin- (via 5-HT<sub>4</sub> receptors), forskolin-, and Mn<sup>2+</sup>-activated AC (Fig. 3) (4,15), indicating that with age, the activity of the catalytic unit of AC decreased. This was accompanied by a slight but significant increase in G<sub>i</sub> proteins, which might contribute to the decline in AC activity (4). Accordingly, in electrically driven right atrial trabecular preparations of these patients, the potency of isoprenaline to increase the force of contraction was about 10-fold higher in the atria from young versus old patients; in these atria, however, the beta-AR density was only marginally altered (4). Similar data of a decreased beta-AR-mediated activation of AC and the inotropic potency of isoprenaline were also obtained in ventricular preparations of the aging human heart (16-18). However, in ventricular myocardium, the beta-AR density and G<sub>s</sub> protein were found to decrease with age, whereas the G<sub>i</sub> protein was unchanged (18). The reason for these (tissue-specific?) differences between the aging atrial and ventricular human myocardium is not known; however, the functional consequence in both tissues is a blunting of beta-AR-mediated effects and all other cAMP-dependent effects with aging. In this respect, the aging human heart resembles the failing human heart, which also exhibits a markedly reduced responsiveness of beta-AR stimulation and all other cAMP-mediated effects (2,3). However, it should be emphasized that decreases in beta-AR and the accompanying increase in G<sub>i</sub> protein are markedly more pronounced in the failing human heart than they are in the aging human heart (2-4).

**Activity of GRK is not altered in the aging human heart.** One important factor that contributes to beta-AR desensitization in failing human hearts is an increase in the activity of cardiac GRK (Fig. 2) (3,5,6). G-protein-coupled receptor kinase phosphorylates the agonist-occupied receptor, thereby facilitating binding of arrestin to the phosphorylated receptor. This leads to uncoupling of the receptor from the G<sub>alpha-s</sub>-AC system and finally to a decrease in the beta-AR response to agonist stimulation. Chronic beta-AR stimulation is a strong trigger mechanism to increase GRK activity (19). In aging, sympathetic activity is also increased, thus leading to chronic stimulation of beta-ARs (8,9). Therefore, also in the aging human heart, an increase in GRK activity might contribute to cardiac beta-AR desensitization. However, the present data clearly show that this is not the case. The activity of neither cytosolic nor membranous GRK was significantly altered in the aging human RA as compared with the atria from children. It should be noted that we recently have shown in several studies that age-dependent differences in the cardiac autonomic receptor systems are more pronounced when data obtained in children are compared with those obtained in elderly patients (>60 years) than when comparing data of middle-aged patients (20 to 40 years) versus elderly patients (>60 years) (4,10,15). Therefore, we are quite sure that

because of the lack of difference in GRK activity between children and elderly patients (>60 years) in this study, there is no age-dependent alteration in GRK activity in the human heart. Moreover, the fact that we found a significant increase in GRK activity in the RA from end-stage failing hearts versus non-failing hearts (as to be expected from the published data) makes it unlikely that we might have overlooked changes in the aging heart. This is further supported by the fact that in the present study we found the well-known decreased activity of basal as well as 10 μmol/l GTP-, 10 μmol/l isoprenaline-, and 10 μmol/l forskolin-stimulated AC in the atrial membranes from elderly patients versus children (4,15).

The reasons for the differences in GRK's regulation in the aging versus failing human heart are not completely understood at present. However, it should be considered that, in aging, the increase in sympathetic activity develops slowly and moderately, as indicated by the observation that plasma noradrenaline levels (often taken as an indirect index of sympathetic activity) (20) increase continuously 10% to 15% per decade due to enhanced spillover of noradrenaline into the circulation (21,22). Thus, with aging, plasma noradrenaline levels are increased up 300 to 600 pg/ml (9,23). In chronic HF, on the other hand, increases in sympathetic activity occur much more rapidly and are more pronounced than in the aging heart (24). Thus, plasma noradrenaline reaches within a rather short time (compared with aging) levels between 600 and 1300 pg/ml (24,25). The GRKs, however, exclusively mediate receptor-specific uncoupling in response to prolonged high concentrations of agonists (26,27). Thus, the time course and intensity of increases in sympathetic activity differ between the aging and failing human heart, and these differences might be a possible explanation for the different regulation of GRKs in the aging and failing human heart.

To the best of our knowledge, this is the first study on possible age-dependent changes in GRK activity in the human heart. However, there was a recent study of the rat heart that also failed to find any age-dependent alterations in cardiac *GRK2* or *GRK5* activity (28).

In the heart, three isoforms of GRK are expressed: *GRK2*, *GRK3*, and *GRK5*, whereby *GRK2* is the most abundant form (3,6,7). Moreover, GRK isoforms phosphorylate rhodopsin with the relative order of potency: *GRK2* >> *GRK3* = *GRK5* (29).

Whereas *GRK2* and *GRK3* activation invariably involves a translocation from the cytosol to the cell membrane (30), which is targeted for *GRK2* by G-protein beta-gamma-subunits, *GRK5* seems to rest constitutively in the membrane and does not undergo translocation (31). In the present study, we found increases in GRK activity only in the cytosol and membranes of the failing human heart; whether this is the sum of changes in different GRKs or just an increase in cytosolic and membrane-translocated *GRK2* we cannot decide from the present experiments, because the phosphorylation assay used in this study cannot distinguish

between the individual subtypes. On the other hand, GRK activity was not changed in either the cytosol or membranes of the aging human heart. Thus, although we cannot exclude the possibility that induction of other GRKs like *GRK3* or *GRK5*, which appear to be unaltered in the failing human heart (6,32), might contribute to the regulation of cytosolic and membranous GRK activity, as assessed in the present study, it is very likely that with our method we have assessed predominantly the activity of right atrial *GRK2*, and this is increased in the failing and unchanged in the aging human heart.

**Study limitations.** In this study, we have tried to find age-dependent changes in children versus elderly patients. Children were quite young (mean age 9 years). At this age, many children were prepubertal, and hence, maturation processes are not finished. In addition, patients were not completely normal but had a mild cardiac diagnosis (NYHA class of the group of elderly patients was <II), which also might have affected our data. We cannot exclude that these facts might have contributed to the lack of age-dependent differences in GRK activity, but it will be rather impossible to obtain tissue samples from really normal human hearts with a more appropriate age distribution. However, in patients with a very similar etiology and age distribution, we have recently found significant correlations between age and right atrial AC activity (4), age and right atrial muscarinic receptors (15), and age and right atrial noradrenaline reuptake transporter (uptake<sub>1</sub>) (10). These findings strongly favor the idea that, in this study, there is indeed a lack of age-dependent changes in right atrial GRK activity.

In addition, in the present study, the lack of age-dependent changes in GRK activity was assessed in the human RA. Whether this also holds true for human left ventricular GRK activity is not known. It should be mentioned that changes in the beta-AR system observed in the human RA can or cannot be identical to those in human ventricular myocardium. Thus, in end-stage HF, beta-ARs were markedly decreased in right atrial and left ventricular preparations of the human heart (33). On the other hand, as discussed earlier in this report, age-dependent changes in beta-ARs appear to be different between atrial (4) and ventricular myocardium (18). However, in the present study, we have found increased GRK activity in the RA from patients with end-stage HF (Fig. 2), and similar GRK increases also occur in the ventricular myocardium of patients with end-stage HF (5,32). This also might be taken as an indication that in the ventricular myocardium of the aging human heart GRK activity is not altered, although the final experimental proof is lacking.

**Conclusions.** In the aging human heart, GRK activity (presumably *GRK2*) is not altered, in contrast to the failing human heart, where *GRK2* is significantly increased. Thus, increased *GRK2* activity seems *not* to contribute to the beta-AR desensitization observed in the aging human heart. Although there are some similarities between the aging and

failing human heart, in this respect, the aging human heart is clearly different from the failing human heart.

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