Overexpression of Cyclic Adenosine Monophosphate Effluent Protein MRP4 Induces an Altered Response to β-Adrenergic Stimulation in the Senescent Rat Heart

Aude Carillion, M.D., Sarah Feldman, Cheng Jiang, M.D., Fabrice Atassi, Ph.D., Na Na, M.D., Nathalie Mougenot, Sophie Besse, Ph.D., Jean-Sébastien Hulot, M.D., Ph.D., Bruno Riou, M.D., Ph.D., Julien Amour, M.D., Ph.D.

ABSTRACT

Background: In the senescent heart, the positive inotropic response to β-adrenoceptor stimulation is reduced, partly by dysregulation of β1- and β3-adrenoceptors. The multidrug resistance protein 4 (MRP4) takes part in the control of intracellular cyclic adenosine monophosphate concentration by controlling its efflux but the role of MRP4 in the β-adrenergic dysfunction of the senescent heart remains unknown.

Methods: The β-adrenergic responses to isoproterenol were investigated *in vivo* (stress echocardiography) and *in vitro* (isolated cardiomyocyte by Ionoptix® with sarcomere shortening and calcium transient) in young (3 months old) and senescent (24 months old) rats pretreated or not with MK571, a specific MRP4 inhibitor. MRP4 was quantified in left ventricular homogenates by Western blotting. Data are mean ± SD expressed as percent of baseline value.

Results: The positive inotropic effect of isoproterenol was reduced in senescent rats *in vivo* (left ventricular shortening fraction 120 ± 16% *vs.* 158 ± 20%, $P < 0.001$, n = 16 rats) and *in vitro* (sarcomere shortening 129 ± 37% *vs.* 148 ± 35%, $P = 0.004$, n = 41 or 43 cells) as compared to young rats. MRP4 expression increased 3.6-fold in senescent compared to young rat myocardium ($P = 0.012$, n = 8 rats per group). In senescent rats, inhibition of MRP4 by MK571 restored the positive inotropic effect of isoproterenol *in vivo* (143 ± 11%, n = 8 rats). *In vitro* in senescent cardiomyocytes pretreated with MK571, both sarcomere shortening (161 ± 45% *vs.* 129 ± 37%, $P = 0.007$, n = 41 cells per group) and calcium transient amplitude (132 ± 25% *vs.* 113 ± 27%, $P = 0.007$) increased significantly.

Conclusion: MRP4 overexpression contributes to the reduction of the positive inotropic response to β-adrenoceptor stimulation in the senescent heart. **(ANESTHESIOLOGY 2015; 122:334-42)**

ELDERLY patients are exposed to a higher mortality risk during the perioperative period.[1] In the senescent heart, diastolic dysfunction[2–4] and a reduced response to β-adrenoceptor stimulation are observed which may contribute to hemodynamic instability during the perioperative period.[5] Down-regulation of β1- and β2-adrenoceptors impair the positive inotropic effect of β-adrenergic stimulation,[2–6] whereas overexpression of β3-adrenoceptor increases nitric oxide production *via* nitric oxide synthase 1, activates protein kinase G, and then promotes the increased hydrolysis of cyclic adenosine monophosphate (cAMP) by activation of phosphodiesterases.[3] However, these abnormalities only partly contribute to the alteration of the β-adrenergic pathway.

A complementary mechanism of regulation of cAMP has been recently described, the multidrug resistance protein 4 (MRP4).[7,8] This channel was first described in resistance to chemotherapy as it allows malignant cells to extrude the drug and survive[9] but further studies revealed its role as transporter of cyclic nucleotides.[10–12] In cardiomyocytes and vascular smooth cells, MRP4 is located at the plasma membrane level[7] and extrudes cAMP.[7–9,11] *In vitro*, inhibition of MRP4 increased intracellular cAMP level and protein kinase A activity,[8] leading to a greater positive inotropic

What We Already Know about This Topic

- The multidrug resistance protein MRP4 plays an important role in regulation of intracellular cyclic adenosine monophosphate and β-adrenergic stimulated heart performance
- Elderly patients are at significant risk of perioperative cardiovascular compromise; however, the role of MRP4 in the senescent heart has never been investigated

What This Article Tells Us That Is New

- MRP4 is overexpressed in the senescent rat heart and is involved in the limited positive inotropic response of the senescent heart to β-adrenergic stimulation
- MRP4 may be a therapeutic target for altering the inotropic reserve of elderly patients

Submitted for publication January 27, 2014. Accepted for publication September 2, 2014. From the Sorbonne Universités UPMC Univ Paris 06, UMR INSERM-UPMC 1166, Paris, France (A.C., S.F., C.J., F.A., N.M., J.-S.H., B.R., J.A.); Department of Anesthesiology and Critical Care Medicine (A.C., J.A.), Department of Emergency Medicine and Surgery (N.N., B.R.), and Department of Pharmacology (J.-S.H.), Hôpital Pitié-Salpêtrière, Assistance Publique-Hôpitaux de Paris, Paris, France; Université de Reims Champagne-Ardenne and Department of Anesthesiology and Critical Care Medicine, Robert Debré Hospital, Reims, France (A.C.); Wuhan University, Wuhan, The People's Republic of China (C.J.); UMR INSERM 902 Université Evry Val d'Essone, Evry, France (S.B.); and Université Paris-Descartes, Paris, France (S.B.).

 Anesthesiology 2015; 122:334-42

effect of β-adrenoceptor stimulation. In mice with genetic deletion of MRP4, an increase in cAMP has been observed in cardiomyocytes after adenylyl cyclase stimulation.[7] However, the involvement of MRP4 in the senescence-induced β-adrenergic dysfunction had not yet been studied.

This study tested the hypothesis that an overexpression of MRP4 contributes to the altered response of the β-adrenoceptor stimulation in the senescent heart.

Materials and Methods

Animals

Experiments were conducted in an authorized laboratory under supervision of an authorized researcher (J. Amour, A-75-20-81). The project has been submitted to the relevant Animal Care Committee through the French Ministry of High Education and Research (Comité Régional d'Ethique en Expérimentation Animale Paris-comité 3, Paris, France). Wistar male rats have been studied in two groups: senescent rats (24 months old) and young adult rats (3 months old). Animals were purchased from Janvier (Le Genest St Isle, France) and cared according to the *Guiding Principles in the Care and Use of Animals* in a labeled housing place (B-75-13-08) with food and water *ad libitum*. The heart was removed during anesthesia after the measurements of arterial blood pressure and weighed, except for the hearts used for the isolation of cardiomyocytes, in order to shorten the time of ischemia. Then, the left ventricle was carefully dissected and frozen in liquid nitrogen for Western blotting experiments.

Stress Echocardiography

Transthoracic stress echocardiography was performed on anesthetized rats under 1 to 2% isoflurane using a General Electric Vivid 7 instrument (Aulnay-sous-Bois, France) as previously described.[3,13,14] Systolic function and inotropy were studied with left ventricular shortening and ejection fractions using a modified version of Simpson's analysis on parasternal short-axis and long-axis views in M mode. These variables were measured in basal conditions and under β-adrenergic stimulation using isoproterenol (10 μg kg^{-1} min^{-1}, continuous intravenous administration) with or without intravenous pretreatment with MK571 (30 mg/kg, Enzo Life Sciences, Villeurbanne, France) a specific inhibitor of MRP4[7,15] or the same volume of NaCl 0.9% as control. Stress values were determined after stabilization of heart rate (HR) 6 min after each drug administration.

Arterial Pressure Measurements

In vivo, at least 2 days after the echocardiographic assessment, the rats were anesthetized using pentobarbital (50 mg/kg intraperitonally) to measure arterial blood pressure. Pressure transducer catheter (size 2F, Millar Micro-tip catheter transducer, model SPR-407; Millar Instruments, Inc., Houston, TX), was introduced into the right carotid artery and connected to a pressure transducer (Gould Electronic, Cleveland, OH). After stabilization, the arterial systolic, mean arterial pressure and diastolic pressures were recorded. From the arterial blood pressure tracings, the HR and the maximum positive values of first derivative of arterial pressure (+dP/dt) were quantified. The cardiac variables were recorded under baseline conditions and under β-adrenergic stimulation using isoproterenol (10 μg kg^{-1} min^{-1}, continuous intravenous administration although the tail vein) with or without pretreatment with MK571 (intravenous administration of 30 mg/kg 6 min before through the tail vein)[7,15] or the same volume of normal saline as a control. Stress values were determined after stabilization of HR.

Measurements of Intracellular Calcium Transient and Contractile Function in Isolated Cardiomyocytes

Ventricular cardiomyocytes were isolated from rat hearts on a Langendorf apparatus using enzymatic digestion by collagenase A (Roche Diagnostics, Meylan, France) as previously described.[16] Under intravenous anesthesia (pentobarbital 65 mg/kg, intraperitonally) the chest was opened, the heart was removed and washed in buffer then connected through the aorta to the Langendorf perfusion cannula. An anterograde perfusion *via* the coronary circulation was applied to the heart with a HEPES buffer (117 mM NaCl, 5.7 mM KCl, 1.5 mM KH_2PO_4, 4.4 mM $NaHCO_3$, 1.7 mM $MgCl_2$, 11.7 mM glucose, 10 mM creatine, 21 mM HEPES, and 20 mM taurine, all form Sigma-Aldrich, L'Isle d'Abeau Chesnes, Saint-Quentin Fallavier, France) bubbled with oxygen and maintained at 37°C and pH 7.40. The cardiac digestion was performed by the perfusion of the same buffer with collagenase A (1.2 to 1.4 mg/ml), 100 μM EGTA and 240 μM $CaCl_2$ (both from Sigma-Aldrich). After 60 to 80 min, the heart was removed and the atria excised. A careful mechanical dissection completed the digestion. The cells were filtered and resuspended in the native calcium-free buffer with bovine serum albumin (Sigma-Aldrich). Calcium was progressively added to the suspension to reach an extracellular calcium concentration of 0.5 mM. Freshly isolated cardiomyocytes were used in the same day. The ventricular cardiomyocytes were loaded for 20 min at room temperature with Fura2-AM (1 μM, Molecular Probes, Invitrogen, Saint-Aubin, France) and then resuspended in the HEPES buffer with 0.5 mM calcium. Contractility and calcium transient of the cardiomyocytes were assessed on each cell with a Ionoptix® platform (Ionoptix Corporation, Milton, MA).[17] Only rod-shaped cardiomyocytes with sharp edges were studied. Cardiomyocytes with spontaneous contraction or sarcolemal blebs were avoided. Myocytes were electrically stimulated at 1 Hz and 8 V in a room at 25°C. The contractile properties of the cardiac myocytes were analyzed with the IonWizard® software (Ionoptix®, Ionoptix Corporation) from the trace of the sarcomere shortening by peak shortening (PS), time to PS normalized to PS and the maximum shortening velocity (–dL/dt) for the shortening phase and time to 90% relengthening and the maximum relengthening velocity (+dL/dt) for the relengthening phase were recorded.

Myocytes were alternatively exposed to a light of 340 and 380 nm by the Fluorescence System Interface with Hyperswitch (Ionoptix Corporation). Fura2-AM is a fluorescent dye whose emission wavelength depends on the calcium concentration and the ratio 340/380 of fluorescence of the Fura2-AM is correlated to the intracellular calcium level.[17] The changes in Fura2-fluorescence intensity (FFI) reflect the variation of the intracellular calcium level during the cardiac cycle, also known as calcium transient. The calcium transient was described in our study by the ΔFFI, amplitude of the FFI increase after twitch (peak FFI-baseline FFI) and the time course of the fluorescence constant decay (tau). The inhibition of MRP4 was provided by a pretreatment with MK571 (10^{-7} M). The contraction and fluorescence ratio of the cardiomyocyte were recorded continuously at basal condition and after adjunction of isoproterenol (10^{-6} M). Since calibration was not performed, only percent changes with isoproterenol and baseline values of ΔFFI could be compared between groups. Stress values were evaluated at maximal cell contraction. As MK571 could also inhibit leukotrienes receptors CysLT1 and CysLT2 and thus influence the response to β-adrenoceptor stimulation, we performed additional experimentation in isolated cardiomyocytes using BAY-u9773 (10^{-7} M) (Cayman Chemical Bertin Pharma, Montigny-le-Bretonneux, France), a specific inhibitor of CysLT1 and CysLT2 receptors.[18] Then, β-adrenoceptor stimulation was performed as described in the experiment with MK571.

Western Blotting

The left ventricles were removed from anesthetized rats and frozen in liquid nitrogen. The samples were homogenated in a lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% triton, phosphatase and protease-inhibitor cocktail, Sigma-Aldrich). The protein concentration was determined by the Bradford protein assay (Bio-Rad, Marnes-la Coquette, France). After denaturation in Laemmli buffer, 60 μg of total protein extract was loaded in each lane for separation in a sodium dodecyl sulfate 9% polyacrylamide electrophoresis gel and transferred on a nitrocellulose membrane (Hybond, Amersham, GE Healthcare, Velizy, France). After saturation in milk, each membrane was incubated overnight at 4°C with primary antibodies (anti-MRP4 1/200: M4I-80 (Abcam, Paris, France), anti-CysLT1 Receptor 1/10,000 or anti-CysLT2 receptor 1/5,000 (both from Cayman Chemical), and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) 1/1,500 (ab9485, Abcam)).[8] The day after, membranes were washed with a Tris-saline buffer with Tween and incubated with appropriate secondary antibody (anti-rat 1/2,500, anti-rabbit 1/2,500, all from Cell Signaling, Ozyme, Saint-Quentin en Yvelines, France). Relative quantification of the targeted protein was achieved by fluorescence recording on EthanDIGE reader with an ECL® detection system (GE Healthcare). MRP4 was detected at 150 kDa and GAPDH at 37 kDa. MRP4 expression was quantified using Image J software (NIH, Bethesda, MA) and normalized *versus* GAPDH expression to ensure no variation in protein gel loading. In addition, Ponceau S staining was performed to confirm that GAPDH expression did not differ between young and senescent rats. Two membranes were generated and comparison was performed on ratio of MRP4/GAPDH expression normalized on the mean expression in control samples in the concerning gel.

Statistical Analysis

Data are expressed as mean ± SD. We used absolute values to compare baseline characteristics between young and senescent rats and delta percent changes from baseline to compare the pharmacological effects, as previously desc ribed.[3,13,14,16,19,20] As a matter of fact, SD of delta percent changes represents the variation of the pharmacological effect we are measuring, whereas the SD of absolute values mainly reflects interindividual differences. Moreover, some variables were expected to significantly differ at baseline because we compared young and senescent rats. The main criteria of our study was sarcomere shortening, especially PS. Assuming a baseline value of PS of 10.7 ± 2.3%, an alpha risk of 0.05 and a beta risk of 0.20, we determined that a sample size of at least n = 35 cells per group would enable us to detect a 15% change in PS (PASS 11 software, Statistical Solutions Ltd., Cork, Ireland). Young and senescent rats or cells were studied alternatively. Experiments could not be blinded because young and senescent rats look particularly different. For *in vivo* experiments, treatment was allocated before anesthesia by manual randomization. For *in vitro* experiments, cells were parted in aliquots. Inhibitor or suspension buffer was added after manual randomization before being examined. Means were compared using the Student *t* test or one-way ANOVA with *post hoc* test Newman–Keuls. All *P* values were two-tailed and a *P* value of less than 0.05 was considered as significant. Statistical analysis was performed using NCSS 7.0 software (Statistical Solutions Ltd.).

Results

We studied a total of 21 young and 20 senescent rats. Two young and four senescent rats exhibited ventricular fibrillation or asystole during invasive arterial pressure measurement and were excluded from the analysis.

Baseline Characteristics

Baseline characteristics of rats are reported in table 1. As expected, senescent rats had significantly higher body weight and heart weight than young rats but the heart to body weight ratio was not significantly different between groups. Using echocardiography, HR in senescent rats was significantly lower in senescent than that in young rats. Both left ventricular shortening and ejection fractions were not significantly different between senescent and young rats. Under baseline conditions *in vivo*, the HR was significantly lower in senescent rats but the mean arterial pressure was

Table 1. Characteristics of Young and Senescent Rats

	Young	Senescent
General characteristics (no. of rats)	(n = 21)	(n = 20)
Body weight (g)	430 ± 60	566 ± 50*
	(n = 12)	(n = 12)
Heart weight (mg)	872 ± 104	1,294 ± 274*
Heart weight/body weight (mg/g)	3.2 ± 0.3	3.1 ± 0.5
Echocardiography (no. of rats)	(n = 16)	(n = 16)
Heart rate (beats/min)	366 ± 33	331 ± 13*
LV ejection fraction (%)	81 ± 7	80 ± 3
LV shortening fraction (%)	47 ± 6	44 ± 4
Arterial catheterization (no. of rats)	(n = 16)	(n = 16)
HR (beats/min)	420 ± 35	355 ± 33*
Systolic arterial pressure (mmHg)	130 ± 16	135 ± 19
Diastolic arterial pressure (mmHg)	105 ± 12	105 ± 16
Mean arterial pressure (mmHg)	113 ± 13	115 ± 17
+dP/dt (mmHg/s)	2,680 ± 501	2,250 ± 665*
Sarcomere kinetics (no. of cells)	(n = 43)	(n = 41)
PS (%)	10.7 ± 2.3	9.5 ± 3.0*
TPS (ms)	11.8 ± 3.0	20.6 ± 9.6*
–dL/dt (mm/s)	–3.0 ± 0.8	–2.1 ± 1.0*
+dL/dt (mm/s)	2.7 ± 1.0	1.7 ± 1.1*
TR90 (ms)	261 ± 83	340 ± 102*
Calcium transient (no. of cells)	(n = 43)	(n = 41)
ΔFFI (arbitrary units)	0.53 ± 0.19	0.37 ± 0.27†
Tau (ms)	124 ± 25	125 ± 28

Data are mean ± SD.

*$P < 0.05$ *vs.* Young. †Because calibration was not performed, these values could not be compared between young and senescent rats.

–dL/dt = maximum shortening velocity; +dL/dt = maximum relengthening velocity; +dP/dt = first derivative of arterial pressure; ΔFFI = changes in Fura-2 fluorescence intensity; HR = heart rate; LV = left ventricular; PS = peak shortening; TPS = time to peak shortening; TR90 = time to 90% relengthening.

not significantly different between groups. *In vitro*, the basal characteristics of sarcomere shortening of the cardiomyocytes revealed a moderate alteration of contractility and relaxation parameters in the senescent cardiomyocytes. The cardiomyocytes from senescent rats displayed reduced PS and –dL/dt and increased time to PS. The +dL/dt was significantly lower and time to 90% relengthening was significantly higher in the senescent cardiomyocytes (table 1).

Effects of MK571, a Specific Inhibitor of MRP4

Since MK571 may also inhibit leukotrienes receptors, we verified that inhibition of CysLT1 and CysLT2 receptors by BAY-u9773 did not significantly modify the response to isoproterenol on sarcomere shortening in isolated cardiomyocytes from young rats (data not shown). Moreover, CysLT1 and CysLT2 receptors protein expression were not significantly different between young and in senescent rats (1.0 ± 0.7 *vs.* 1.0 ± 0.8 arbitray units, $P = 0.99$ and 1.0 ± 0.8 *vs.* 1.0 ± 0.7 arbitray units, $P = 0.90$, respectively).

Administration of saline did not significantly modify any echocardiographic variables in the senescent or young groups. In senescent rats, administration of MK571 had no significant effect on any echocardiographic variable. In contrast, in young rats, a slight but significant increase in left ventricular shortening and ejection fractions was observed (table 2). During arterial catheterization, administration of saline or MK571 induced no significant effect on HR, mean arterial pressure, or dP/dt in any group. Nevertheless, in young rats during echocardiography, the isoproterenol-induced increase in HR seems to be blunted by MK571 (table 2), an effect not observed during arterial catheterization (table 2). We did not test the direct effect of MK571 on sarcomere shortening and calcium transient since isolated cells were incubated with or without MK571. Nevertheless, we did not observe any significant difference in the groups incubated with or without MK571 (table 2).

Effects of MK571 on β-Adrenoceptor Stimulation

In vivo, using echocardiography, the positive inotropic effect of β-adrenoceptor stimulation was altered in senescent rats as compared with young rats. Pretreatment with MK571 restored the positive inotropic effect in the senescent group to the level observed in young rats (fig. 1).

During arterial catheterization, the increase in HR and the decrease in mean arterial pressure induced by isoproterenol were not significantly different between senescent and young rats. Pretreatment with MK571 restored the positive inotropic effect in the senescent group to the level observed in young rats (fig. 1).

In vitro, the senescent cardiomyocytes exhibited a reduced positive inotropic response to isoproterenol compared with young cardiomyocytes (fig. 2). Pretreatment with MK571 restored the positive inotropic response of sarcomere shortening in senescent cardiomyocytes returning to the level observed in young cardiomyocytes, whereas it had no significant effect in young cardiomyocytes (table 2). Calcium transient increased with β-adrenoceptor stimulation. In senescent group, calcium transient increase was more important in cardiomyocytes pretreated with MK571 than in non-pretreated cardiomyocytes (fig. 3).

Expression of MRP4

In senescent rats, MRP4 protein expression increased 3.6-fold in comparison to young rats ($P = 0.012$) (fig. 4). Using Ponceau staining, we confirmed the absence of significant variation in GAPDH expression in senescent compared to young rats (203 ± 9 *vs.* 211 ± 15 arbitrary units, $P = 0.70$)

Discussion

In the current study, we confirmed that the positive inotropic response to β-adrenoceptor stimulation is altered in senescent hearts. We observed that the expression of MRP4 was 3.6-fold increased in left ventricle of senescent rats and inhibition of MRP4 by MK571 restored the positive inotropic effect of β-adrenoceptor stimulation in senescent rats both *in vivo* and *in vitro*. In parallel, the calcium transient was improved by MK571 pretreatment in the senescent cardiomyocytes. Consequently, these results strongly support the role of MRP4 in the altered β-adrenergic response in the senescent heart.

In vivo, our results confirm that the systolic function was preserved (left ventricular shortening and ejection

Table 2. Comparison of the Response to β-Adrenergic Stimulation in Young and Senescent Rats with or without Pretreatment by MK571

	Baseline	Saline	Isoproterenol	Baseline	MK	Isoproterenol
	Young			Young with MK571		
Echocardiography (no. of rats)	(n = 8)			(n = 8)		
HR (beats/min)	361 ± 40	358 ± 20	421 ± 30*	371 ± 28	358 ± 17	387 ± 29
LV ejection fraction (%)	79 ± 9	78 ± 7	96 ± 3*	83 ± 5	87 ± 5*	97 ± 2*
LV shortening fraction (%)	46 ± 6	45 ± 7	72 ± 8*	47 ± 5	53 ± 8*	71 ± 5*
Arterial catheterization (no. of rats)	(n = 6)			(n = 4)		
HR (beats/min)	440 ± 17	450 ± 33	530 ± 23*	389 ± 33	384 ± 41	525 ± 50*
Mean arterial pressure (mmHg)	116 ± 15	110 ± 11	61 ± 8*	109 ± 11	107 ± 12	60 ± 8*
+dP/dt (mmHg/s)	2,800 ± 566	2,600 ± 860	6,733 ± 1,115*	2,500 ± 383	2,933 ± 787	5,800 ± 1,649*
Sarcomere kinetics (no. of cells)	(n = 43)				(n = 41)	
PS (%)	10.7 ± 2.3	NA	15.4 ± 2.5	NA	9.5 ± 3.0	15.3 ± 2.4
TPS (ms)	11.8 ± 3.0		6.9 ± 1.7		20.6 ± 9.6	7.0 ± 2.6
–dL/dt (mm/s)	–3.0 ± 0.8		–5.1 ± 1.5		–2.1 ± 1.0	–5.5 ± 2.2
+dL/dt (mm/s)	2.7 ± 1.0		3.7 ± 1.4		1.7 ± 1.1	3.9 ± 1.3
TR90 (ms)	261 ± 83		214 ± 83		340 ± 102	211 ± 59
	Senescent			Senescent with MK571		
Echocardiography (no. of rats)	(n = 8)			(n = 8)		
Heart rate (beats/min)	327 ± 13	322 ± 10	333 ± 13	335 ± 12†	320 ± 23	341 ± 21
LV ejection fraction (%)	81 ± 4	80 ± 3	89 ± 3	78 ± 2	79 ± 3	92 ± 3
LV shortening fraction (%)	46 ± 4	44 ± 3	54 ± 4	42 ± 2	43 ± 2	61 ± 5
Arterial catheterization (no. of rats)	(n = 4)			(n = 4)		
HR (beats/min)	351 ± 30	376 ± 19	424 ± 36*	360 ± 33	360 ± 33	446 ± 19*
Mean arterial pressure (mmHg)	118 ± 16	113 ± 28	73 ± 44*	112 ± 20	113 ± 30	71 ± 13*
+dP/dt (mmHg/s)	1,950 ± 661	2,150 ± 412	3,070 ± 1,429*	2,550 ± 598	2,750 ± 1,370	4,900 ± 744*
Sarcomere kinetics (no. of cells)	(n = 41)				(n = 41)	
PS (%)	9.5 ± 3.0	NA	11.7 ± 3.3†	NA	8.8 ± 2.9	13.2 ± 2.3
TPS (ms)	20.6 ± 9.6		14.1 ± 5.7†		20.9 ± 8.3	11.0 ± 3.2
–dL/dt (mm/s)	–2.1 ± 1.0		–3.0 ± 1.3†		–1.8 ± 0.8	–3.7 ± 1.2
+dL/dt (mm/s)	1.7 ± 1.1		1.9 ± 1.1†		1.7 ± 1.2	2.6 ± 1.5
TR90 (ms)	340 ± 102		316 ± 65†		316 ± 67	290 ± 74

Data are mean ± SD. Δ is % of baseline value.

*$P < 0.05$ *vs.* baseline; †$P < 0.05$ *vs.* Young.

–dL/dt = maximum shortening velocity; +dL/dt = maximum relengthening velocity; +dP/dt = first derivative of arterial pressure; ΔFFI = changes in Fura-2 fluorescence intensity; HR = heart rate; LV = left ventricular; NA = not applicable; PS = peak shortening; TPS = time to peak shortening; TR90 = time to 90% relengthening.

fractions) in senescent hearts, as previously observed.[3] *In vitro*, the cardiomyocytes extracted from senescent rats exhibited an impaired sarcomere shortening with prolonged duration of shortening (time to peak shortening) and time to 90% relengthening consistent with the literature.[2,4] The positive inotropic response to β-adrenoceptor stimulation was reduced in senescent rats both *in vivo* and *in vitro*. The senescent heart displays different mechanisms to preserve itself from an excessive work such as the reduction of positive chronotropic and inotropic effects of the β-adrenergic stimulation. β-Adrenergic dysfunction may contribute to long-term saving of heart function and senescence adaptation. Unfortunately during the perioperative period, this β-adrenergic dysfunction limits cardiac output adaptation and favors hemodynamic instability in aging patients.[3] Although β-blockers have been shown to improve long-term survival in patients with chronic heart failure and may be also beneficial during the perioperative period, deleterious effects have also been clearly demonstrated in the perioperative period when bleeding or postoperative complications occur.[21]

Several modifications in the β-adrenergic signaling pathway have been described in the senescent heart.[2,4] Due to decreased expression of β1- and β2-adrenoceptors, less cAMP is produced in the senescent heart after isoproterenol. The effect of direct stimulation of adenylyl cyclase is also reduced, but the effect of a cAMP analog is preserved, confirming the crucial role of intracellular cAMP level in the β-adrenergic response.[6] β3-adrenoceptor induces the production of nitrite oxide by nitrite oxide synthase 1 that activates protein kinase G which increases the catabolism of cAMP by phosphodiesterase activation.[3] An increase in β3-adrenoceptor expression and activity in the senescent heart[3] contributes to the reduction of the positive inotropic effect of β-adrenergic stimulation as we previously observed in the diabetic cardiomyopathy.[13]

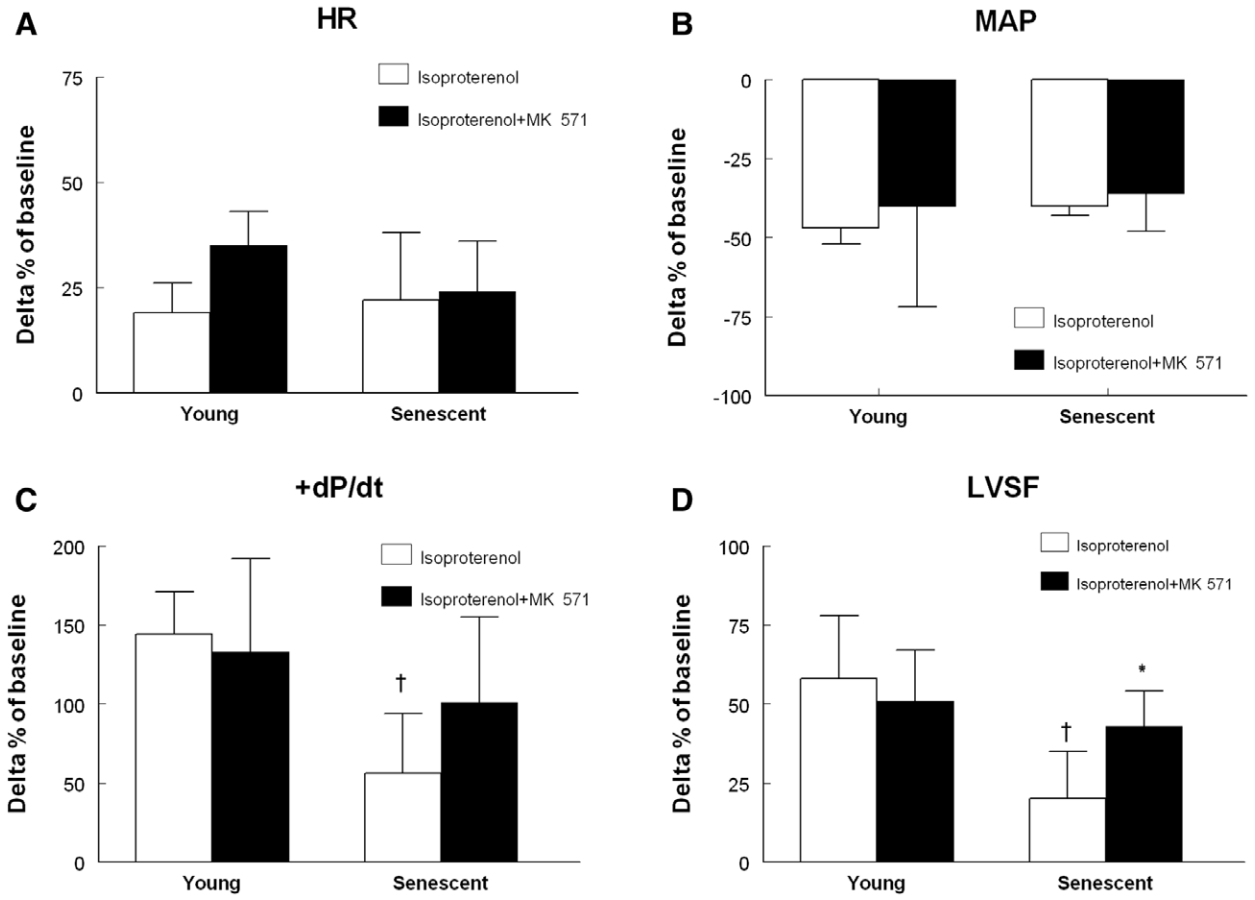

Fig. 1. Comparison of the effect of isoproterenol on heart rate (HR) (*A*), mean arterial blood pressure (MAP) (*B*), positive first derivative for maximal rates of arterial pressure development (+dP/dt) (*C*), and left ventricular shortening fraction (LVSF) (*D*) in young and senescent rats with and without MK571. HR, MAP, and +dP/dt were obtained during invasive catheterization (n = 6 and 4, respectively), and LVSF was obtained during echography (n = 8 in each group). Data are mean ± SD. **P* < 0.05 *versus* group without MK571; †*P* < 0.05 *versus* Young group.

The transmembrane protein MRP4 is known as an effluent pump of cyclic nucleotides in platelets,[22] hepatic,[9] and renal cells.[23] MRP4 is overexpressed in the liver of female aging mice.[24] In vascular smooth cells[8,11,25] and cardiomyocytes,[7] MRP4 is known to control the efflux of cAMP. In cultured cells, MRP4 could regulate the submembrane pool of cAMP by efflux and interaction with phosphodiesterases.[10] In this context, the large MRP4 overexpression observed in senescent rats suggests a pathway for increased removal from the myocyte of cAMP that was synthesized after β-adrenoceptor stimulation. Such cAMP elimination from the cell could markedly decrease the positive inotropic effect of β-adrenergic stimulation.

MK571 has been used as a specific inhibitor of MRP4.[7,8,11,12] The effect of MK571 is rapid and constant after intravenous administration.[15,26] Inhibition of MRP4 by MK571 induces an elevation in cAMP intracellular concentration in vascular smooth cells[8,11] and cardiomyocytes.[12] Combined inhibition of MRP4 and phosphodiesterases induces an elevation of cAMP more important than with isolated inhibition of MRP4 suggesting that the two mechanisms may be additive.[11] Inhibition of MRP4 by MK571 enhanced the chronotropic effect of isoproterenol in neonatal mice cardiomyocytes.[12] In our study, MK571 had no or little effect on *in vivo* or *in vitro* contractility before β-adrenergic stimulation. This result is consistent with the fact that MK571 is not a direct agonist of β-adrenoceptors and MRP4 only affects submembrane compartment of cAMP produced after β-adrenergic stimulation.[7] The elevation of cAMP after isoproterenol stimulation of isolated cardiac myocytes from 9-month-old mice is increased by silencing of MRP4.[7] In agreement with these results, we demonstrated here that MRP4 inhibition restored the positive inotropic effect of β-adrenoceptors stimulation both *in vivo* and *in vitro* in the senescent rats. MRP4 may extrude the cAMP produced by β-adrenoceptor stimulation by isoproterenol and may limit the increase in intracellular second messenger concentration. The intracellular calcium level closely reflects the contractility of a muscle as it is directly involved in the actin/myosin interaction and muscle shortening. The β-adrenergic stimulation induces a positive inotropic effect *via* cAMP production which activates the protein kinase A. Activation of protein

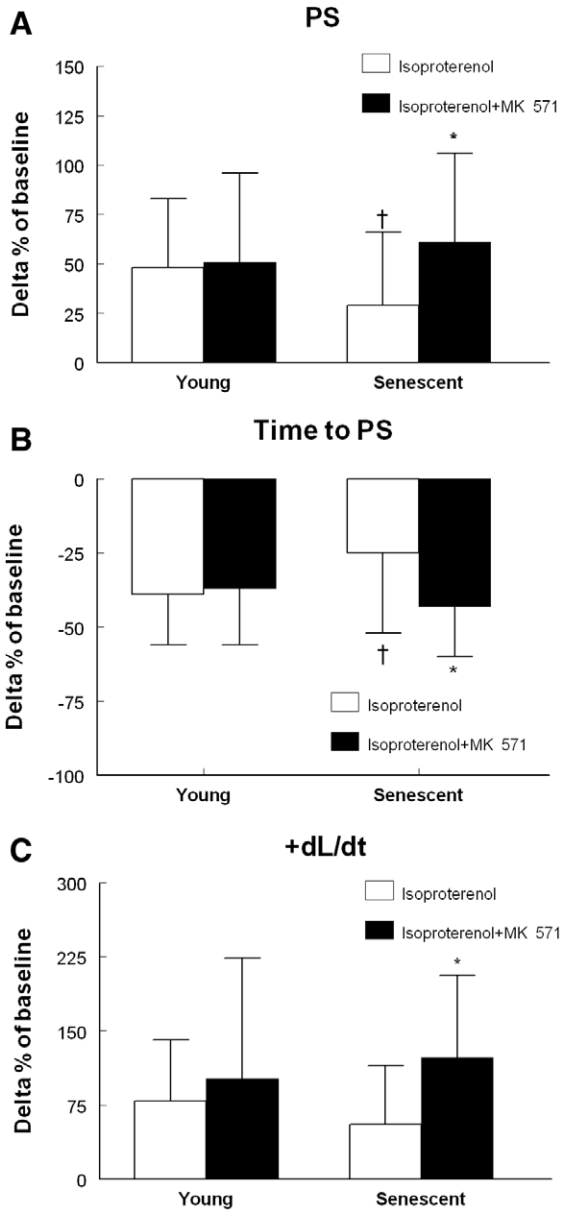

Fig. 2. Sarcomere kinetics: comparison of the effect of isoproterenol on the peak shortening (PS) amplitude (*A*), time to PS (*B*), and maximum velocity of shortening (–dL/dt) (*C*) in young and senescent rats with and without MK571 (n = 41 to 43 cells in each group). Data are mean ± SD. **P* < 0.05 *versus* group without MK571; †*P* < 0.05 *versus* Young group.

kinase A increases calcium transient after phosphorylation of targeted proteins (calcium channel, ryanodine receptor, sarco-endoplasmic reticulum ATPase, and troponin). Since calcium transient was altered in senescent cardiomyocytes and since we observed an increase in calcium transient amplitude and sarcomere shortening induced by β-adrenergic stimulation after MK571 pretreatment, we can conclude that MRP4 overexpression plays a role in the altered inotropic response of β-adrenergic stimulation in the senescent heart.

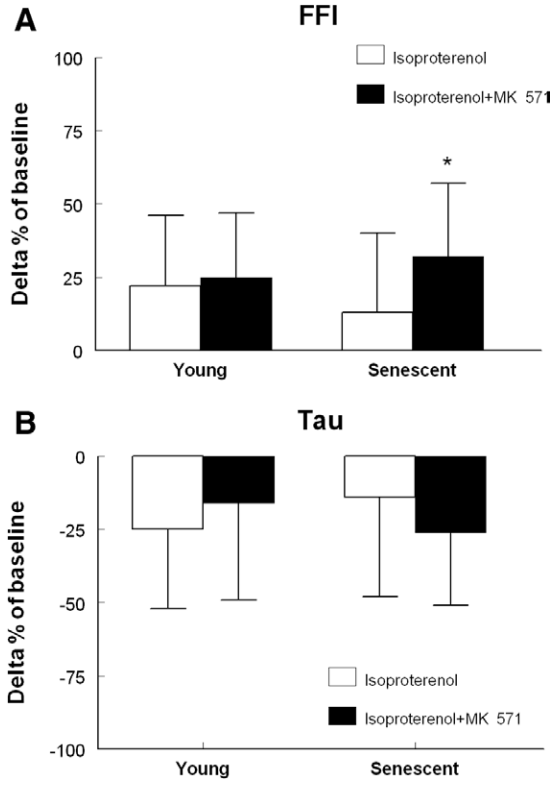

Fig. 3. Calcium transient: comparison of the effect of isoproterenol (10^{-6} M) on the amplitude of calcium transient (changes in Fura2-fluorescence intensity [ΔFFI]) (*A*) and time decay constant of the calcium transient (tau) (*B*) in isolated cardiomyocytes from young and senescent rats with and without MK571 (n = 41 to 43 cells in each group). Data are mean ± SD.

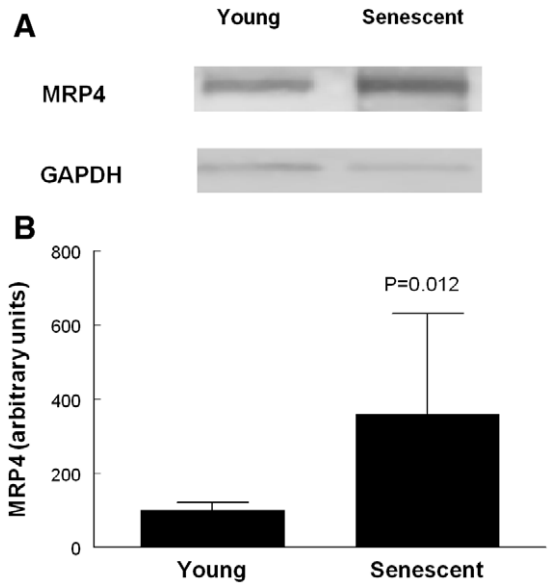

Fig. 4. Representative Western blot (*A*) and normalized densitometric data (*B*) showing left ventricular expression of multidrug resistance protein 4 (MRP4) compared with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in young and senescent rats (n = 8 in each group). Data are mean ± SD.

The following points have to be considered to assess the clinical relevance of our results. First, these experiments were conducted in rats and the results may not be generalized to humans as rat myocardium exhibits several differences with human myocardium, including MRP4 expression and function.[27] Further experiments are mandatory to confirm the increased expression and the role of MRP4 in the human senescent heart. Second, *in vivo* studies require anesthetized animals. We used isoflurane inhalation for echocardiography and pentobarbital for arterial blood pressure recordings. Halogenated agents are known to interfere with β-adrenergic stimulation in different cardiomyopathies,[19,20] but our results are consistent, regardless of the anesthetic technique used. Since the isoproterenol-induced increase in HR seems to be blunted by MK571 in young rats during echography (*i.e.*, with isoflurane) but not during arterial catheterization (*i.e.*, with pentobarbital), we cannot rule out the hypothesis that an interaction between MK571 and baroreflex activity was differentially altered by anesthetics *in vivo*. Third, *in vitro* experiments may be affected by a selection bias as cardiomyocytes surviving to cell isolation may differ from the more disabled ones present in the total heart. Fourth, we elected not to directly measure cAMP or cyclic guanosine monophosphate concentrations since these messenger molecules are compartmentalized within the cell, and average cellular concentrations may not reflect critical concentrations at near relevant kinase mediators within the cell. Furthermore, MRP4 has been located in caveolae near from β-adrenoceptors and we think it could effectively act on this pool of cAMP, not necessarily on the whole cell cAMP mean concentration.[7] Fifth, we used MK571 to selectively inhibit MRP4. This drug is also an inhibitor of the CysLT1receptor for leukotrienes D4.[15,28] The inhibition of CysLT1 and CysLT2 receptors by MK571 could alleviate the negative inotropic effect due to leukotrienes D4 in heart and be confusing in interpretation of our results.[17,29] However, inhibition of CysLT1 and CysLT2 receptors by BAY-u9773 did not modify the response to isoproterenol in young cardiomyocytes and CysLT1 and CysLT2 receptor expressions were not significantly modified in senescent hearts. In the same manner, that we cannot rule out the possibility that the *in vivo* effects of MK571 could also be modulated by CysLT1 receptors in vascular smooth muscle.[30] Sixth, in a therapeutic view, chronic inhibition of MRP4 may alter other organ function or may be compensated by an increase in phosphodiestereases.

In conclusion, we observed that MRP4 is overexpressed in the heart of senescent rats and plays an important role in the altered positive inotropic response to β-adrenoceptor stimulation.

Acknowledgments

The authors thank David Baker, D.M., F.R.C.A. (Department of Anesthesiology and Critical Care, Hôpital Necker-Enfants Malades, Paris, France), for reviewing the manuscript and Anne-Marie Lompré, Ph.D. (UMR INSERM 1166, Paris, France), for critical reading of the manuscript.

Dr. Carillion was the recipient of a research grant from the Fondation pour la Recherche Médicale (Paris, France; FRM grant no. DEA20090616244). Dr. Jiang was the recipient of a doctoral grant from the People's Republic of China (State Scholarship Fund by China Scholarship Council; grant no. 201206270014).

Competing Interests

The authors declare no competing interests.

Correspondence

Address correspondence to Dr. Carillion: Département d'Anesthésie-Réanimation, Groupe hospitalier Pitié-Salpêtrière, 47–83 Boulevard de l'Hôpital, 75651 Paris cedex 13, France. aude.carillion@psl.aphp.fr.

References

1. Gajdos C, Kile D, Hawn MT, Finlayson E, Henderson WG, Robinson TN: Advancing age and 30-day adverse outcomes after nonemergent general surgeries. J Am Geriatr Soc 2013; 61:1608–14
2. Lakatta EG: Arterial and cardiac aging: Major shareholders in cardiovascular disease enterprises: Part III: Cellular and molecular clues to heart and arterial aging. Circulation 2003; 107:490–7
3. Birenbaum A, Tesse A, Loyer X, Michelet P, Andriantsitohaina R, Heymes C, Riou B, Amour J: Involvement of beta 3-adrenoceptor in altered beta-adrenergic response in senescent heart: Role of nitric oxide synthase 1-derived nitric oxide. ANESTHESIOLOGY 2008; 109:1045–53
4. Schmidt U, del Monte F, Miyamoto MI, Matsui T, Gwathmey JK, Rosenzweig A, Hajjar RJ: Restoration of diastolic function in senescent rat hearts through adenoviral gene transfer of sarcoplasmic reticulum Ca(2+)-ATPase. Circulation 2000; 101:790–6
5. Stratton JR, Cerqueira MD, Schwartz RS, Levy WC, Veith RC, Kahn SE, Abrass IB: Differences in cardiovascular responses to isoproterenol in relation to age and exercise training in healthy men. Circulation 1992; 86:504–12
6. Xiao RP, Tomhave ED, Wang DJ, Ji X, Boluyt MO, Cheng H, Lakatta EG, Koch WJ: Age-associated reductions in cardiac beta1- and beta2-adrenergic responses without changes in inhibitory G proteins or receptor kinases. J Clin Invest 1998; 101:1273–82
7. Sassi Y, Abi-Gerges A, Fauconnier J, Mougenot N, Reiken S, Haghighi K, Kranias EG, Marks AR, Lacampagne A, Engelhardt S, Hatem SN, Lompre AM, Hulot JS: Regulation of cAMP homeostasis by the efflux protein MRP4 in cardiac myocytes. FASEB J 2012; 26:1009–17
8. Hara Y, Sassi Y, Guibert C, Gambaryan N, Dorfmüller P, Eddahibi S, Lompré AM, Humbert M, Hulot JS: Inhibition of MRP4 prevents and reverses pulmonary hypertension in mice. J Clin Invest 2011; 121:2888–97
9. Sampath J, Adachi M, Hatse S, Naesens L, Balzarini J, Flatley RM, Matherly LH, Schuetz JD: Role of MRP4 and MRP5 in biology and chemotherapy. AAPS PharmSci 2002; 4:E14
10. Xie M, Rich TC, Scheitrum C, Conti M, Richter W: Inactivation of multidrug resistance proteins disrupts both cellular extrusion and intracellular degradation of cAMP. Mol Pharmacol 2011; 80:281–93

11. Sassi Y, Lipskaia L, Vandecasteele G, Nikolaev VO, Hatem SN, Cohen Aubart F, Russel FG, Mougenot N, Vrignaud C, Lechat P, Lompré AM, Hulot JS: Multidrug resistance-associated protein 4 regulates cAMP-dependent signaling pathways and controls human and rat SMC proliferation. J Clin Invest 2008; 118:2747–57
12. Sellers ZM, Naren AP, Xiang Y, Best PM: MRP4 and CFTR in the regulation of cAMP and β-adrenergic contraction in cardiac myocytes. Eur J Pharmacol 2012; 681:80–7
13. Amour J, Loyer X, Le Guen M, Mabrouk N, David JS, Camors E, Carusio N, Vivien B, Andriantsitohaina R, Heymes C, Riou B: Altered contractile response due to increased beta3-adrenoceptor stimulation in diabetic cardiomyopathy: The role of nitric oxide synthase 1-derived nitric oxide. ANESTHESIOLOGY 2007; 107:452–60
14. Amour J, Loyer X, Michelet P, Birenbaum A, Riou B, Heymes C: Preservation of the positive lusitropic effect of beta-adrenoceptors stimulation in diabetic cardiomyopathy. Anesth Analg 2008; 107:1130–8
15. Eppihimer MJ, Russell J, Anderson DC, Epstein CJ, Laroux S, Granger DN: Modulation of P-selectin expression in the postischemic intestinal microvasculature. Am J Physiol 1997; 273(6 pt 1):G1326–32
16. Biais M, Jouffroy R, Carillion A, Feldman S, Jobart-Malfait A, Riou B, Amour J: Interaction of metabolic and respiratory acidosis with α and β-adrenoceptor stimulation in rat myocardium. ANESTHESIOLOGY 2012; 117:1212–22
17. Ren J: Attenuated cardiac contractile responsiveness to insulin-like growth factor I in ventricular myocytes from biobreeding spontaneous diabetic rats. Cardiovasc Res 2000; 46:162–71
18. Liu P, Misurski DA, Gopalakrishnan V: Cysteinyl leukotriene-dependent $[Ca^{2+}]_i$ responses to angiotensin II in cardiomyocytes. Am J Physiol Heart Circ Physiol 2003; 284:H1269–76
19. Amour J, David JS, Vivien B, Coriat P, Riou B: Interaction of halogenated anesthetics with alpha- and beta-adrenoceptor stimulations in diabetic rat myocardium. ANESTHESIOLOGY 2004; 101:1145–52
20. Vivien B, David JS, Hanouz JL, Amour J, Lecarpentier Y, Coriat P, Riou B: The paradoxical positive inotropic effect of sevoflurane in healthy and cardiomyopathic hamsters. Anesth Analg 2002; 95:31–8
21. Le Manach Y, Collins GS, Ibanez C, Goarin JP, Coriat P, Gaudric J, Riou B, Landais P: Impact of perioperative bleeding on the protective effect of β-blockers during infrarenal aortic reconstruction. ANESTHESIOLOGY 2012; 117:1203–11
22. Jedlitschky G, Tirschmann K, Lubenow LE, Nieuwenhuis HK, Akkerman JW, Greinacher A, Kroemer HK: The nucleotide transporter MRP4 (ABCC4) is highly expressed in human platelets and present in dense granules, indicating a role in mediator storage. Blood 2004; 104:3603–10
23. Yacovino LL, Aleksunes LM: Renal efflux transporter expression in pregnant mice with type I diabetes. Toxicol Lett 2012; 211:304–11
24. Fu ZD, Csanaky IL, Klaassen CD: Effects of aging on mRNA profiles for drug-metabolizing enzymes and transporters in livers of male and female mice. Drug Metab Dispos 2012; 40:1216–25
25. Cheng D, Ren J, Jackson EK: Multidrug resistance protein 4 mediates cAMP efflux from rat preglomerular vascular smooth muscle cells. Clin Exp Pharmacol Physiol 2010; 37:205–7
26. Depré M, Margolskee DJ, Hsieh JY, Van Hecken A, Buntinx A, De Lepeleire I, Rogers JD, De Schepper PJ: Plasma drug profiles and tolerability of MK-571 (L-660,711), a leukotriene D4 receptor antagonist, in man. Eur J Clin Pharmacol 1992; 43:427–30
27. Russel FG, Koenderink JB, Masereeuw R: Multidrug resistance protein 4 (MRP4/ABCC4): A versatile efflux transporter for drugs and signalling molecules. Trends Pharmacol Sci 2008; 29:200–7
28. Wong WS, Zhu H, Liao W: Cysteinyl leukotriene receptor antagonist MK-571 alters bronchoalveolar lavage fluid proteome in a mouse asthma model. Eur J Pharmacol 2007; 575:134–41
29. Feurstein G: Leukotrienes and the cardiovascular system. Prostaglandins 1984; 27:781–92
30. Mazzetti L, Franchi-Micheli S, Nistri S, Quattrone S, Simone R, Ciuffi M, Zilletti L, Failli P: The ACh-induced contraction in rat aortas is mediated by the Cys Lt1 receptor *via* intracellular calcium mobilization in smooth muscle cells. Br J Pharmacol 2003; 138:707–15