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Upregulation of BAD, a pro-apoptotic protein of the BCL2 family, in vascular smooth muscle cells exposed to uremic conditions

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ABSTRACT

Chronic kidney disease (CKD) has recently emerged as a major risk factor for cardiovascular pathology. CKD patients display accelerated atherosclerotic process, leading to circulatory complications. However, it is currently not clear how uremic conditions accelerate atherosclerosis. Apoptosis is an important homeostatic regulator of vascular smooth cells under pathological conditions. In the present study, we explored the regulation of apoptosis in cells of the vascular wall in the uremic context. We analysed the expression and regulation of the proteins of the BCL2 family that play an essential role in apoptosis. Our results, obtained in mice and primary human smooth muscle cells exposed to two uremic toxins, point to the existence of an alteration in expression and function of one pro-apoptotic member of this family, the protein BAD. We explore the regulation of BAD by uremic toxins and report the sensitization of vascular smooth muscle cells to apoptosis upon BAD induction.

1. Introduction

Chronic kidney disease (CKD) is a frequent medical condition in Europe and North America [1]. CKD can occur either as a complication of various pathological states, such as diabetes and hypertension, or as a result of direct kidney lesions, for example induced by glomerulonephritis. While end stage-renal disease was until recently considered to be the most critical determinant of the health outcome, it is now known that cardiovascular disease represents a much larger source of mortality in these patients [1]. The CKD population presents a nearly 10-fold increase in the risk of cardiovascular disease-associated mortality [2,3]. Research performed over the past decade points to CKD as a potential factor favoring the appearance of atherosclerosis and its progression, although the mechanisms implicated remain essentially unknown [4]. CKD might alter the arterial wall via some well-identified atherosclerosis risk factors, such as inflammation or metabolic alterations [5,6]. At the cellular level, CKD also promotes the accumulation of various uremic toxins, such as inorganic phosphate (Pi) or urea. Whether these toxins directly exert a deleterious action on cells of the vascular wall remains however essentially unknown [7].

Apoptosis is a homeostatic response that plays an important role in the regulation of cells in the normal and pathological arterial wall [8,9]. While apoptosis is usually considered to be a form of silent cell death that is followed by the quick elimination of cell remnants, several recent studies point to a complex role in atherosclerosis [9]. Macrophages have attracted most of the attention as target cells for apoptosis in the course of atherosclerosis [10,11]. The possible role of vascular smooth muscle cells (SMC) was recently examined using transgene technology used to express a SM22α-human diphtheria toxin receptor in mice [12]. In this model, diphtheria toxin treatment affords selective and controlled induction of arterial SMC apoptosis and the exploration of its impact *in vivo*. In this setting, Clarke et al. showed that SMC apoptosis has profound consequences on atherosclerosis [12,13]. Apoptosis does not only promote the early stages of atherogenesis and the appearance of lesions, but it can also influence the progression of lesions that are already constituted [12,13]. Apoptosis of arterial SMC could for example determine the mineral composition of the plaque as well as its stability [12–14].

Apoptosis is a form of programmed cell death that is under a strong control by the mitochondria and the proteins of the BCL2 family [15]. This family of proteins consists of approximately 20 members exerting pro- or anti-apoptotic effects, and sharing one or multiple domains of homology (BCL2-Homology, or BH domain)

Abbreviations: CKD, chronic kidney disease; SMC, smooth muscle cells; Pi, inorganic phosphate; ox-LDL, oxidized low density lipoproteins; 7-KC, 7-ketocholesterol.

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[15]. BCL2 proteins exert a key role in the execution of apoptosis, through their ability to permeabilize the mitochondrial outer membrane and to promote the cytosolic release of pro-apoptotic factors, such as cytochrome c [15]. Although BCL2 proteins play an essential role in apoptosis regulation and execution, it is still unclear if there are some alterations of their expression and function in CKD. In the present study, we performed a comprehensive survey of the expression of BCL2 proteins in an animal model of CKD and in human aortic SMC exposed *in vitro* to uremic conditions. Our findings point to the existence of a selective alteration in the expression of BAD, a pro-apoptotic protein of the BCL2 family, in uremic conditions. We explore the possible functional consequences of this alteration.

2. Material and methods

2.1. Animals

C57/BL-6 mice from Charles Rivers Laboratories (Lyon, France) were rendered uremic according to Massy et al. [16], using a procedure described in the Supplementary materials and methods.

2.2. Cell culture and reagents

Culturing of human aortic SMC is described in the Supplementary materials and methods. ABT-737 was a gift from Abbott. All other reagents were from Sigma.

2.3. Immunoblots and quantitative PCR analysis

Immunoblots and quantitative PCR analysis were performed using standard procedures (see Supplementary materials and methods for details).

2.4. Determination of BAD protein stability

Cycloheximide, a blocker of protein translation, was used to analyze the protein half life of BAD, as previously described [17]. Cycloheximide was incubated at a concentration of 50 μM, and HASMC were treated for 1 h as indicated, before analysing the total protein extracts for their content of BAD.

2.5. Statistical analyses

Immunoblots were scanned and quantified using the software ImageJ (NIH, http://rsbweb.nih.gov/ij/). Wilcoxon unpaired test and Student's *t*-test were used as indicated, and a value of $p < 0.05$ was considered as threshold for significance.

3. Results

3.1. Alterations in the expression of BCL2 proteins in the arterial wall of uremic mice

In order to examine the expression of BCL2 proteins in the arterial wall in uremic conditions, we randomly assigned 20 WT C57/Bl6 mice into 2 groups: the first group consisting of uremic mice ($n = 10$), and the second, control group with mice in non-uremic conditions ($n = 10$). After 6 weeks of uremia, all animals were sacrificed and biochemical measurements were performed in order to verify the respective status of the animals (Table 1). Aortic tissues were dissected and protein samples were prepared and analyzed by immunoblotting to survey the expression of proteins of the BCL2 family (Fig. 1). In these extracts, we detected some of the proteins of the BCL2 family. Some anti-apoptotic (BCL2, BCL-XL) and some pro-apoptotic members of the BCL2 family (BAK, BAX, BAD) were detectable at the protein level (data not shown). Interestingly, most BCL2 proteins were present at comparable levels between control and uremic samples, with the noticeable exception of BAD, a pro-apoptotic member of the BH3-only subgroup, whose levels were found to be increased by approximately 2-fold in uremic compared to control aortas (Fig. 1). BAD exerts its apoptosis-sensitizing function by neutralizing the anti-apoptotic proteins BCL2 and BCL-XL [18]. To estimate the imbalance in the expression of BCL2 proteins in uremic mice, we measured the ratio between BAD and BCL-XL and found the same 2-fold increase (Fig. 1).

Table 1
Effect of chronic renal failure on basic plasma parameters of mice[a].

	Sham ($n = 10$)	Uremic ($n = 10$)	p[b]
Urea (mM)	8.9	27.2	<0.001
Creatinine (μM)	18.5	57.0	<0.001

[a] Data are median values measured at the time of animal sacrifice.
[b] p Value measured with Wilcoxon test.

3.2. Urea increases the expression of BAD in human aortic SMC in cultures

Aortic tissues consist of several cell types, including the SMC. To explore the role that uremic toxins might play in the up-regulation of BAD, we exposed human aortic SMC in culture to two well-established uremic toxins, inorganic phosphate (Pi) and urea. Both toxins were applied at concentrations considered to be relevant in CKD [7]: Pi was applied at a concentration of 4 mM, and urea at 20 mM on SMC for 24 h (Fig. 2). Under conditions of exposure of SMC to urea, we noticed an overexpression of BAD resulting in an increase in the BAD/BCL-XL ratio (Fig. 2A). These variations were noticed independently of any effect on cell viability (data not shown). BAD is a protein characterized by its rapid turn-over, mainly due to proteasomal degradation [17,19,20]. We decided to explore the mechanisms through which BAD might be induced by measuring its mRNA levels (Fig. 2B) and its protein turn-over (Fig. 2C). We did not detect any change in the levels of BAD mRNA in SMC exposed to urea (Fig. 2B). Conversely, we observed an approximately 50% extension of the half-life for BAD in human aortic SMC treated with urea *in vitro* (Fig. 2C).

3.3. BAD is an apoptotic sensitizer in human aortic SMC

Having noticed that BAD was overexpressed through post-transcriptional mechanisms in SMC exposed to urea, we decided to explore the potential consequences of its increased expression in these cells. An increase in the level of oxidative stress is believed to be a key element in the pathogenesis of uremia [21]. Oxidized lipoproteins, and in particular oxidized low-density lipoproteins (ox-LDL) are well-known pathogenic agents involved in the progression of atherosclerosis in general, and their role as pro-apoptotic species is well established [22]. To examine the sensitivity of aortic SMC to apoptosis, we exposed these cells to 7-ketocholesterol (7-KC), one of the most abundant products of cholesterol oxidation detected in atherosclerotic plaques with a potent pro-apoptotic activity [22]. As previously reported by other investigators, we found that 7-KC induces cell death in SMC (Fig. 3). Upon overnight exposure of these cells to 7-KC, approximately 25% of cells presented chromatin condensation, a typical feature of apoptosis, and a vacuolated aspect. The swollen vacuolar compartment most likely derived from endosomes and/or lysosomes, since the vacuoles could be decorated with the marker Lamp1 (Fig. 3A). When aortic SMC were exposed to urea, a strong synergy was no-

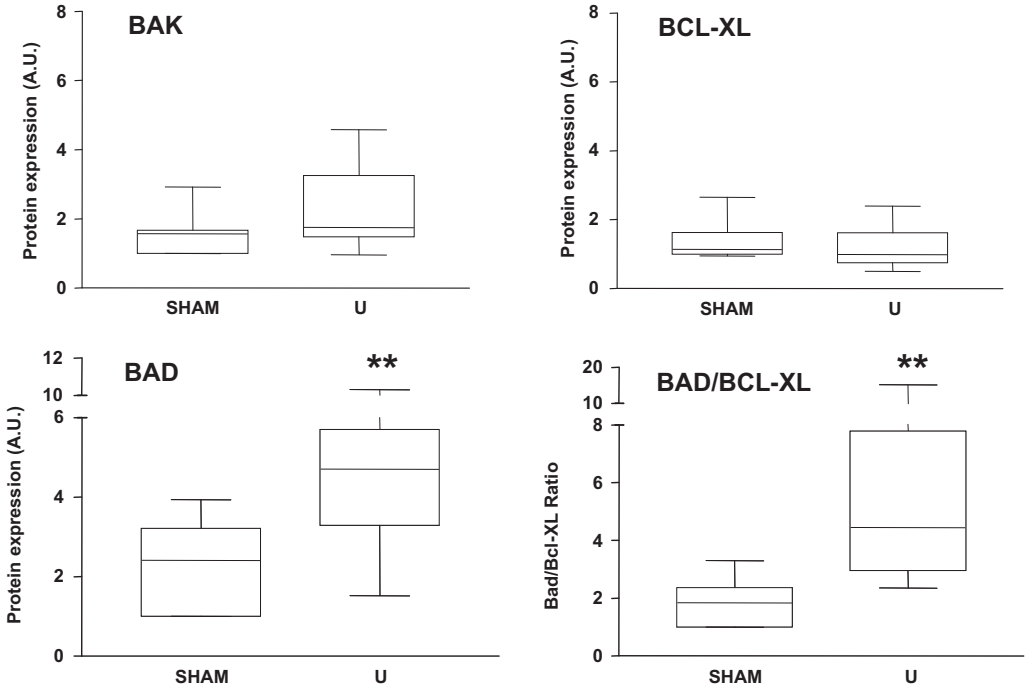

Fig. 1. Analysis of the expression of proteins of the BCL2 family in uremic mice. Protein samples obtained from the aorta of C57/Bl6 mice, rendered uremic (U, $n = 10$) or control (sham, $n = 10$), were used for the analysis of the expression of various members of the BCL2 family by immunoblotting. The results are presented as box plots drawn from the densitometric analysis of the main proteins detected, with the results being expressed in arbitrary units (AU). *indicates $p < 0.005$ compared with sham using the Wilcoxon test.

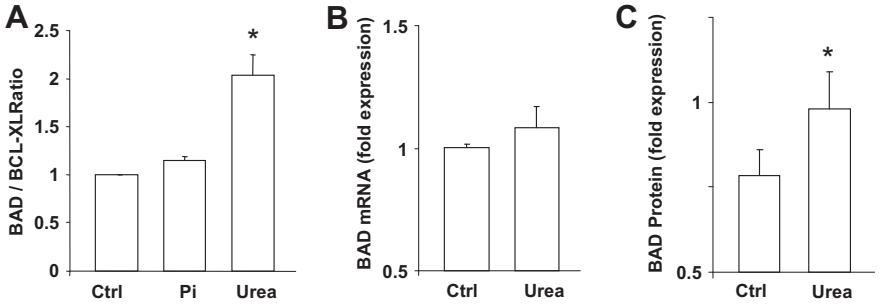

Fig. 2. Regulation of BAD expression in human aortic SMC cells in uremic conditions. (A) BAD protein regulation in aortic SMC exposed to uremic toxins. Results are from three independent experiments analysing the expression of BAD by immunoblotting, after normalizing the data as BAD/BCL-XL ratios. Cells were exposed for 24 h to the indicated uremic toxins, applied at clinically relevant concentrations (Pi 4 mM, urea 20 mM). *for $p < 0.05$ with Student's t-test. (B) Expression of BAD mRNA in aortic SMC exposed to urea. RNA samples were extracted from cells treated as previously described and BAD mRNA was analysed by Quantitative PCR. (C) BAD protein stability. Aortic SMC were treated for 30 min with 50 μM cycloheximide and maintained for 1 h in control or uremic conditions (20 mM urea). Results are mean ± SD from three independent experiments.

ticed with 7-KC, both in terms of vacuolation and apoptosis. A more than twofold increase in the % of cells with chromatin condensation was found upon counting (Fig. 3B). To examine the possibility that the increase in BAD expression that we had previously reported might have played a role in this apoptosis induction, we compared the effects of urea with those of ABT-737, a chemical compound inhibiting the anti-apoptotic proteins of the BCL2 proteins [23], and recently reported by us to induce an efficient stabilization of BAD by interfering with its turn-over, including in SMC [17]. We applied the compound ABT-737 on SMC in cultures, and verified that BAD was induced to the same extent as in SMC treated with urea (data not shown). Interestingly, ABT-737 and urea sensitized aortic SMC to 7-KC-induced apoptosis to the same extent (Fig. 3B).

4. Discussion

Apoptosis of the arterial cells is a key event in vascular remodelling and the progression of atherosclerosis [8,9,12,13]. CKD has been recently identified as a condition that accelerates atherosclerosis [4], but little is still known about the impact of uremia on the regulation of apoptosis in the arterial smooth muscle cells. Here, we explored the regulation of BCL2 proteins, which are core components of the apoptotic machinery, in aortic SMC placed under uremic conditions. We found that BAD, a pro-apoptotic member of the BH3-only subset of this family, is overexpressed in animal tissues and in cells exposed to uremic conditions. In agreement with what is known about the mode of action of BAD, that sensitizes cells to apoptosis through its ability to neutralize the anti-

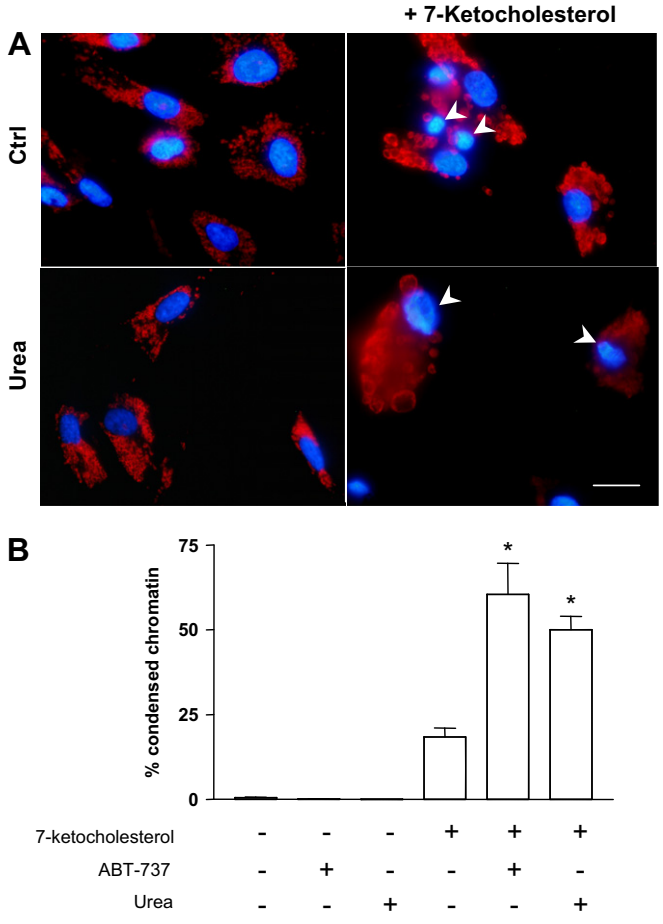

Fig. 3. BAD overexpression sensitizes aortic SMC to 7-ketocholesterol (7-KC)-induced apoptosis. (A) Aortic SMC grown in control conditions or in the presence of 20 mM urea were treated with 50 μM 7-KC and processed and examined with fluorescence microscopy. Cells are labelled with the marker Lamp-1 (red) and with DAPI (blue). Arrow heads indicate condensed chromatin characteristic of apoptotic cells. Bar: 20 μm. (B) Percentage of cells with chromatin condensation. Aortic SMC exposed to urea or ABT-737 (10 μM) were treated with 7-KC and the % of cells with chromatin condensation was determined. The results are average values obtained from three independent countings of approximately 250 cells, each time analyzed from random microscopic fields. $^{*}p < 0.05$ using Student's *t*-test.

apoptotic proteins BCL2 and BCL-XL [18], we found that the induction of BAD by uremic toxins alone did not induce apoptosis but sensitized cells to the pro-apoptotic effect of oxidized cholesterol, a physiologically relevant inducer of this form of programmed cell death [22]. Interestingly, the role of BAD as an apoptotic sensitizer was previously reported in vascular SMC, although in a different context [19]. Altogether, these results suggest that the induction of BAD that we report here might at least partially account for the increased levels of apoptosis that are reported in arteries of uremic patients [14].

Among the family of BCL2 proteins, BAD possesses an original regulation. BAD is usually presented as a signalling node that integrates essential transduction pathways regulating apoptosis [18]. In particular, BAD defines a point of convergence for several kinase cascades that regulate this protein post-translationally. Pro-survival kinases, such as the RAF-MEK-ERK cascade, can neutralize BAD by phosphorylating some key residues and regulate its association with the proteins of the 14-3-3 family and the availability of its BH3 domain to interact with BCL2 or BCL-XL. Recently, we and others have identified BAD as a labile protein whose stability is post-translationally regulated by the proteasome [17,19,20,24]. The findings that we report here confirm these observations and suggest that uremia mainly alters BAD expression through the modulation of this protein's stability. However, details of the mechanisms through which uremic conditions modulate BAD expression will await future investigations, since we did not identify any important alteration in the kinase cascades phosphorylating BAD (data not shown).

CKD is characterized by the retention of many uremic toxins that interfere with cell physiology [7]. Interestingly, we found that urea was the most potent uremic toxin able to induce the expression of BAD in HASMC. While urea was until recently considered to be of little pathological importance in uremia, a recent work points to its contribution to the alterations in the mitochondrial oxidative metabolism that are noticed in uremic conditions [25]. Our findings are therefore in agreement with the emerging notion that urea might be an important uremic toxin in CKD, and one that promotes mitochondrial alterations [25]. In this respect, our observations are centred on the sensitivity of eukaryotic cells to apoptosis, but it will be interesting to explore the contribution of BAD to the different facets of the mitochondrial metabolism. Recent observations indicate that BAD regulates the carbohydrate metabolism in addition to apoptosis [18,26,27]. These observations constitute a strong incentive to further explore the role and the regulation of BAD in the arterial wall in normal and uremic conditions.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.11.144.

References

[1] W.G. Couser, G. Remuzzi, S. Mendis, M. Tonelli. The contribution of chronic kidney disease to the global burden of major noncommunicable diseases. Kidney Int. 2011 [Epub ahead of print].
[2] D.S. Keith, G.A. Nichols, C.M. Gullion, J.B. Brown, D.H. Smith, Longitudinal follow-up and outcomes among a population with chronic kidney disease in a large managed care organization, Arch. Intern. Med. 164 (2004) 659–663.
[3] C.P. Wen, T.Y. Cheng, M.K. Tsai, Y.C. Chang, H.T. Chan, S.P. Tsai, et al., All-cause mortality attributable to chronic kidney disease: a prospective cohort study based on 462,293 adults in Taiwan, Lancet 371 (2008) 2173–2182.
[4] T.B. Drüeke, Z.A. Massy, Atherosclerosis in CKD: differences from the general population, Nat. Rev. Nephrol. 12 (2010) 723–735.
[5] S. Swaminathan, S.V. Shah, Novel inflammatory mechanisms of accelerated atherosclerosis in kidney disease, Kidney Int. 80 (2011) 453–463.
[6] P. Libby, P.M. Ridker, G.K. Hansson, Progress and challenges in translating the biology of atherosclerosis, Nature 473 (2011) 317–325.
[7] R. Vanholder, R. De Smet, G. Glorieux, et al., Review on uremic toxins: classification, concentration, and interindividual variability, Kidney Int. 63 (2003) 1934–1943.
[8] M.R. Bennett, G.I. Evan, S.M. Schwartz, Apoptosis of human vascular smooth muscle cells derived from normal vessels and coronary atherosclerotic plaques, J. Clin. Invest. 95 (1995) 2266–2274.
[9] M.R. Bennett, Life and death in the atherosclerotic plaque, Curr. Opin. Lipidol. 21 (2010) 422–426.

[10] I. Tabas, Apoptosis and plaque destabilization in atherosclerosis: the role of macrophage apoptosis induced by cholesterol, Cell Death Differ. 11 (2004) S12–S16.
[11] T. Seimon, I. Tabas, Mechanisms and consequences of macrophage apoptosis in atherosclerosis, J. Lipid Res. 50 (Suppl) (2009) S382–S387.
[12] M.C. Clarke, N. Figg, J.J. Maguire, A.P. Davenport, M. Goddard, T.D. Littlewood, M.R. Bennett, Apoptosis of vascular smooth muscle cells induces features of plaque vulnerability in atherosclerosis, Nat. Med. 12 (2006) 1075–1080.
[13] M.C. Clarke, T.D. Littlewood, N. Figg, J.J. Maguire, A.P. Davenport, M. Goddard, M.R. Bennett, Chronic apoptosis of vascular smooth muscle cells accelerates atherosclerosis and promotes calcification and medial degeneration, Circ. Res. 102 (2008) 1529–1538.
[14] R.C. Shroff, R. McNair, N. Figg, J.N. Skepper, L. Schurgers, A. Gupta, et al., Dialysis accelerates medial vascular calcification in part by triggering smooth muscle cell apoptosis, Circulation 118 (2008) 1748–1757.
[15] J.C. Martinou, R.J. Youle, Mitochondria in apoptosis: Bcl-2 family members and mitochondrial dynamics, Dev. Cell. 21 (2011) 92–101.
[16] Z.A. Massy, O. Ivanovski, T. Nguyen-Khoa, J. Angulo, D. Szumilak, N. Mothu, et al., Uremia accelerates both atherosclerosis and arterial calcification in apolipoprotein E knockout mice, J. Am. Soc. Nephrol. 16 (2005) 109–116.
[17] Z. Ezzoukhry, C. Louandre, C. François, Z. Saidak, C. Godin, J.C. Mazière, A. Galmiche, The Bcl-2 homology domain 3 (BH3) mimetic ABT-737 reveals the dynamic regulation of Bad, a proapoptotic protein of the Bcl-2 family, by Bcl-xL, Mol. Pharmacol. 79 (2011) 997–1004.
[18] N.N. Danial, BAD: undertaker by night, candyman by day, Oncogene 27 (2008) S53–S70.
[19] W.Z. Ying, H.G. Zhang, P.W. Sanders, EGF receptor activity modulates apoptosis induced by inhibition of the proteasome of vascular smooth muscle cells, J. Am. Soc. Nephrol. 18 (2007) 131–142.
[20] J. Fueller, M. Becker, A.R. Sienerth, A. Fischer, C. Hotz, A. Galmiche, C-RAF activation promotes BAD poly-ubiquitylation and turn-over by the proteasome, Biochem. Biophys. Res. Commun. 370 (2008) 552–556.
[21] Z.A. Massy, P. Stenvinkel, T.B. Drueke, The role of oxidative stress in chronic kidney disease, Semin. Dial. 22 (2009) 405–408.
[22] R. Salvayre, N. Auge, H. Benoist, A. Negre-Salvayre, Oxidized low-density lipoprotein-induced apoptosis, Biochim. Biophys. Acta. 1585 (2002) 213–221.
[23] T. Oltersdorf, S.W. Elmore, A.R. Shoemaker, R.C. Armstrong, D.J. Augeri, B.A. Belli, et al., An inhibitor of Bcl-2 family proteins induces regression of solid tumours, Nature 435 (2005) 677–681.
[24] A. Galmiche, Z. Ezzoukhry, C. François, C. Louandre, C. Sabbagh, E. Nguyen-Khac, et al., BAD, a proapoptotic member of the BCL2 family, is a potential therapeutic target in hepatocellular carcinoma, Mol. Cancer Res. 8 (2010) 1116–1125.
[25] M. D'Apolito, X. Du, H. Zong, A. Catucci, L. Maiuri, T. Trivisano, et al., Urea-induced ROS generation causes insulin resistance in mice with chronic renal failure, J. Clin. Invest. 120 (2010) 203–213.
[26] N.N. Danial, C.F. Gramm, L. Scorrano, C.Y. Zhang, S. Krauss, A.M. Ranger, et al., BAD and glucokinase reside in a mitochondrial complex that integrates glycolysis and apoptosis, Nature 424 (2003) 952–956.
[27] N.N. Danial, L.D. Walensky, C.Y. Zhang, C.S. Choi, J.K. Fisher, A.J. Molina, et al., Dual role of proapoptotic BAD in insulin secretion and beta cell survival, Nat. Med. 14 (2008) 144–153.