Inhibition of AMPK signalling by doxorubicin: at the crossroads of the cardiac responses to energetic, oxidative, and genotoxic stress

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Aims
Cardiotoxic side effects of anthracyclines, the most widely used anticancer drugs, are well documented, while mechanisms involved are not fully elucidated. The cellular energy sensor and regulator AMP-activated protein kinase (AMPK) was suggested as a putative mediator of cardiotoxicity of doxorubicin, the leading anthracycline drug, by our earlier work. Here, we study the interference of doxorubicin with AMPK signalling and potentially involved mechanisms.

Methods and results
Effects of doxorubicin on cell signalling are studied in isolated Langendorff-perfused Wistar rat hearts and in hearts from doxorubicin-treated Wistar rats. In both models, doxorubicin induces energetic, oxidative, and genotoxic stress. Despite energy depletion and unaffected AMPK upstream signalling, doxorubicin does not activate the AMPK pathway and even reduces basal phosphorylation of AMPK and its downstream target acetyl-CoA carboxylase. In contrast, oxidative and genotoxic stress do activate pro-survival mitogen-activated protein kinase (MAPK) and Akt pathways, the latter via DNA-dependent protein kinase activation triggered by DNA damage. Combined inhibition of AMPK and activation of Akt and MAPK lead to activation of growth-stimulating mammalian target of rapamycin (mTOR) signalling.

Conclusion
Our results suggest that in the doxorubicin-challenged heart, a combined energetic, oxidative, and genotoxic stress elicits a specific, hierarchical response where AMPK is inhibited at least partially by the known negative cross-talk with Akt and MAPK pathways, largely triggered by DNA damage signalling. Although such signalling can be protective, e.g. by limiting apoptosis, it primarily induces a negative feedback that increases cellular energy deficits, and via activation of mTOR signalling, it also contributes to the pathological cardiac phenotype in chronic doxorubicin toxicity.

Keywords
Adenosine 5′-monophosphate-activated protein kinase • Anthracyclines • Cardiac energetics • Cardiac signalling • Cardiotoxicity • MK2206

1. Introduction
Anthracyclines, in particular doxorubicin (DXR), are among the most effective and commonly used anticancer drugs. However, a serious side effect of anthracycline therapy is severe cardiotoxicity. This represents a burden to the public health system, given the constantly increasing number of cancer patients and cancer survivors suffering from cardiovascular side effects of chemotherapy.¹ Despite 40 years of research, elucidation of anthracycline cardiotoxicity still remains a challenge in terms of involved mechanisms and prevention. Earlier results by us² and more recently by others³,⁴ gave first evidence for an inhibition of the protein kinase signalling pathway via tumour...
suppressor LKB1 and AMP-activated protein kinase (AMPK) as a potential cardiotoxic mechanism. AMPK is an emerging central sensor and regulator in cellular energy and nutrient signalling.\(^5\)\(^6\) The kinase is activated by numerous stimuli, mainly cellular energy stress during physiological (exercise) or pathological (ischaemia) situations, leading to a rise in AMP/ATP and ADP/ATP ratios, intracellular calcium, and reactive oxygen and nitrogen species. In addition, it is also activated by extracellular signals, such as orexigenic/anorexigenic hormones or cytokines.\(^5\)\(^7\) The activation mechanism consists of an allosteric component (AMP, ADP, or ATP binding to the gamma subunit) and a covalent component (phosphorylation of Thr172 in the alpha subunit controlled by upstream kinases, LKB1 or CamKK\(\beta\), and phosphatases).\(^5\)\(^6\) Once activated, AMPK induces adaptation of energy metabolism through regulation of cellular signalling cascades, activity of metabolic key enzymes, and gene transcription. For example, activated AMPK stimulates ATP-generating processes, including uptake and oxidation of glucose and fatty acids, and suppresses a number of ATP-consuming processes, such as protein synthesis, cellular growth, and proliferation. The latter is mainly mediated by inhibition of mammalian target of rapamycin (mTOR) within the mTORC1 complex.\(^8\)\(^9\)

Several recent studies indicate a more general relationship between a reduced LKB1→AMPK signalling and cardiac disease.\(^8\)\(^10\)\(^11\) In this context, the aim of the present study was to analyse the effect of DXR (i) on cardiac signalling up- and downstream of AMPK, in particular mTOR, and (ii) on Akt/PKB and mitogen-activated protein kinase (MAPK) signalling, both reported to cross-talk with AMPK and to mediate also upstream signals for mTOR.\(^7\) In two different rat cardiotoxicity models, the isolated perfused heart and a more chronic in vivo model, we show that DXR induces combined energy, oxidative, and genotoxic stress. This combined stress leads to inhibition of AMPK without negatively affecting different parameters of AMPK upstream signalling. It also activates MAPK and Akt signalling, e.g. via DNA-damage sensing DNA-PK, and this in turn would negatively impact on AMPK activation. Downstream, this would result in continued ATP consumption without compensating ATP generation, and most importantly in continuous activation of growth-stimulating mTOR, both potentially contributing to the cardiotoxic phenotype.

2. Methods

2.1 Materials

Doxorubicin hydrochloride (DXR) was purchased from Fluka (Buchs, Switzerland). A stock solution (10 mM) was prepared in water and kept frozen until use. Further dilutions were prepared in Krebs–Henseleit buffer just before heart perfusion or in saline before injection to rats. ATP, ADP, and AMP were purchased from Sigma (Saint Louis, MO, USA), protease inhibitor cocktail tablets from Roche (Mannheim, Germany), and phosphatase inhibitor cocktail from Pierce (Rockford, IL, USA). MK2206 (Active Biochemicals, Hong Kong) was first dissolved in DMSO (28 mM, pH 5.75) at 1 mL/min flow rate and 30°C. ATP, ADP, and AMP eluted at 3, 5, and 9 mL, respectively. Elution peaks were integrated with the STAR software (Varian, France).

Total genomic DNA was isolated with the QIAamp DNA mini kit (Qiagen). Nuclear and mitochondrial DNA damage was evaluated using a two-step strategy based on a long PCR and a real-time PCR.\(^14\) A detailed description of reagents, primers, and PCR conditions is available as Supplementary material online. Oxidative modification of proteins was probed by immunodetection of carbonyl groups in the protein side chains with the OxyBlot™ Kit (Millipore, Temecula, CA, USA).

2.2 Rat heart perfusion and animal treatment

Perfusion experiments were essentially performed according to the protocol described earlier.\(^7\) Briefly, hearts of male Wistar rats (~300 g) anaesthetized with sodium pentobarbital (50 mg/kg i.p.) and heparinized (1500 IU/kg i.v.) were quickly removed after checking the depth of anaesthesia (pedal reflex, muscle relaxation) and perfused at constant pressure in a non-circulating Langendorf apparatus without (control) or with 5 μM or 25 μM DXR during 80 min (after initial 30 min period of stabilization with the Krebs–Henseleit buffer) and functional parameters were recorded. In some experiments, Akt inhibitor MK2206 (1 μM) was added to the perfusion medium after 15 min of stabilization period. At the end of perfusion, hearts were rapidly freeze-clamped in liquid nitrogen and stored at −80°C. For the animal cardiotoxicity model, 6-week-old male Wistar rats (~175 g) were anaesthetized with an intramuscular injection of ketamine (50 mg/kg)/xylazine (10 mg/kg) and cathe-terized via the jugular vein.\(^12\) Adequacy of anaesthesia was carefully monitored by regular checking depth of anaesthesia (as above) and physiological parameters (heart and respiratory rate). Anaesthetic support such as fluid supplementation and external heating as well as monitoring and support during anaesthetic recovery was provided. DXR (2 mg/kg) or an equivalent volume of saline was administered to animals via the implanted catheter three times a week during 2 weeks for a total dose of 12 mg/kg. The first injection was realized on the day of cathe-terization. Four weeks after the last injection, rats were anaesthetized again and left ventricular function was measured with a Millar pressure-volume system.\(^13\) Subsequently, the hearts were rapidly removed, freeze-clamped in liquid nitrogen, and stored at −80°C. All procedures involving animals conformed to Directive 2010/63/EU and were approved by the Grenoble Ethics Committee for Animal Experimentation (Com-Eth 15_LBFA-U884-HD-01).

2.3 Quantification of nucleotides, DNA damage, and protein oxidation

Freeze-clamped hearts were homogenized in 0.6 N perchloric acid in liquid nitrogen, centrifuged (12 min, 3000 g, 4°C), neutralized by addition of 5 M potassium carbonate, and centrifuged again (12 min, 3000 g, 4°C). Protein-free extract (30 μL) was separated on a C18 HPLC column (Polaris SC18-A, 250×4.6 Repl, Varian, France) in pyrophosphate buffer (28 mM, pH 5.75) at 1 mL/min flow rate and 30°C. ATP, ADP, and AMP eluted at 3, 5, and 9 mL, respectively. Elution peaks were integrated with the STAR software (Varian, France).

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2.4 Immunoblotting and immunoprecipitation

SDS–PAGE separation of heart homogenates and immunoblotting was performed according to the standard procedures.\(^15\) Detailed descriptions including sources and dilutions of antibodies as well as the immunoprecipitation protocol are available as Supplementary material online.

2.5 Statistics

If not stated otherwise, data are presented as means ± SD. Statistical significance was evaluated by two-tailed Student’s t-test using Sigma Plot. \(*P < 0.05, **P < 0.01, ***P < 0.001.

3. Results

3.1 Impaired cardiac function associated with energetic, oxidative, and genotoxic stress

Two different models of DXR cardiotoxicity have been used throughout this study, representative for acute and chronic toxicity. Acute
effects were analysed in isolated rat hearts perfused during 80 min with 5 or 25 μM DXR, the lower concentration corresponding to DXR concentrations found in plasma of patients after bolus injection.16 Chronic effects were analysed in hearts of rats that received six intravenous injections of DXR (2 mg/kg) over a 2-week period and were sacrificed 4 weeks after the end of the treatment. The latter model has been shown to adequately mimic chronic myocardial alterations in patients.16 Both models were first analysed for cardiac function and markers of different forms of stress and apoptotic status (Figures 1 and 2, see Supplementary material online, Table S1). In isolated hearts, DXR perfusion led to dose-dependent loss of cardiac function (Figure 1A–F and see Supplementary material online, Figure S1), together with an increase in AMP/ATP ratios (Figure 1G) and nuclear and mitochondrial DNA lesions (Figure 1J and K), indicating energetic and genotoxic stress. Significant differences were obtained for almost all parameters at 25 μM DXR, except for protein oxidation as revealed by carbonyl formation (Figure 1H and I) which was expected to increase due to higher superoxide levels described earlier.17 Apoptosis was not yet induced, since PARP cleavage was not detectable (Figure 1L). In the in vivo animal model, DXR treatment reduced weight gain of the animals, mainly during the treatment period (Figure 2A). Four weeks after the end of the treatment, DXR-treated rats also showed impaired heart function (Figure 2B–E) and significantly diminished cardiac ATP levels (Figure 2F). Protein carbonyls were increased (Figure 2G and H), in contrast to isolated heart, thus both energetic and oxidative stress
were present. Nuclear DNA damage was less pronounced and below statistical significance: the lack of detectable lesions under this exposure paradigm is likely to depend on the action of the DNA repair machinery occurring between doxorubicin treatment stage and specimens' analysis. Accordingly, more signs of DNA damage were found in mtDNA (Figure 2I) which, indeed, is known to be repaired far less efficiently when compared with nuclear DNA.14 The onset of apoptosis was clearly revealed by PARP cleavage (Figure 2J). This complete characterization of our models for acute and chronic DXR cardiotoxicity is consistent with earlier reports.2,18,19

3.2 Doxorubicin inhibits the AMPK pathway
AMPK is a key cellular energy sensor and regulator. A hallmark of its molecular function is activation by a fall in the cellular energy state as described by an increased [AMP]/[ATP] and [ADP]/[ATP] ratio.5,7 This activation is crucial for a multitude of compensatory responses aiming at a preservation of cellular energetics and avoiding apoptosis.

However, in both model systems examined here, AMPK was not activated by DXR (Figure 3A and B), despite the obvious energy stress induced (Figures 1G and 2F). On the contrary, basal AMPK phosphorylation at Thr172 was even diminished by DXR. This decrease in AMPK activity was also observed at the level of one of its important downstream targets, acetyl-CoA-carboxylase (ACC), providing an independent readout for full AMPK activity (Figure 3A and B). These data clearly confirm and expand our initial observation on an inhibitory effect of DXR on cardiac AMPK activity2 that later has been reproduced by others.3,4

3.3 Doxorubicin does not affect signalling upstream of AMPK
To elucidate potential mechanisms of AMPK inhibition, we first focused on its upstream signalling in the isolated heart (Figure 3C and D), but similar data for most parameters were obtained for the animal model (see Supplementary material online, Figure S2). Protein levels of AMPK kinases were generally unchanged by DXR.
including LKB1, the by far most important upstream kinase in the heart, and calcium calmodulin-dependent protein kinase kinase (CamKKα and β).20 Only CamKKα was reduced at 25 μM DXR, but this isoform is not considered bona fide upstream kinase in vivo.20 We further examined whether LKB1 regulation was altered. Although this kinase is mostly considered to be constitutively active,20,21 some studies correlated LKB1 phosphorylation at Ser42822 or deacetylation at Lys4823 with its downstream activity. In particular, SIRT1 was proposed to deacetylate nuclear LKB1, resulting in LKB1 export into the cytosol and activation of cytosolic AMPK.23 Our data revealed no DXR-induced changes in SIRT1 levels, but an increase in both deacetylation of LKB1 (Figure 3D) and in vivo phosphorylation of LKB1 (see Supplementary material online, Figure S2), which should both rather favour cytosolic LKB1 activity. Another mechanism for LKB1 inhibition suggested recently is adduct formation with HNE (4-hydroxy-2-nonenal), a byproduct of lipid peroxidation during oxidative stress in the heart.11 However, the amount of HNE-protein adducts was not increased by DXR (see Supplementary material online, Figure S3). Finally, phosphatases are emerging as important players in controlling the AMPK activation state. We examined the activity of phosphatase PP2C, which can dephosphorylate AMPK in vitro.20,21 This activity was unaltered by DXR in perfused heart (see Supplementary material online, Figure S4). Collectively, we could not find evidence for an altered upstream signalling to be responsible for the AMPK inhibition by DXR.

3.4 Doxorubicin activates the Akt pathway

An alternative mechanism to down-regulate AMPK activity may be a cross-talk with other signalling pathways. One of the most relevant candidates in the heart is Akt, as AMPK inhibition by the Akt pathway has been reported by us and others24–27 and was detected in heart pathologies.28 Most recent data indicate that Akt may be also activated by genotoxic stress as a substrate of DNA-dependent protein kinase (DNA-PK). The DNA-PK is itself activated by DNA double-strand breaks,29,30 a typical consequence of DXR action. In line with the genotoxic stress detected (Figure 1J and K), the activating phosphorylation of DNA-PK was readily increased after DXR treatment in both of our models (Figure 4A and B). Notably, even though measured DNA damage did not reach significance in isolated heart at 5 μM DXR (Figure 1J and K) or the animal model (Figure 2I, see also comments under section 3.1), an important phosphorylation of DNA-PK was observed (Figure 4A and B). Consistently, DXR

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Figure 4 Activation of DNA-PK – Akt and MAPK pathways by doxorubicin and negative Akt-AMPK cross-talk. Phosphorylated and total proteins of the DNA-PK–Akt pathway (A and B) in perfused rat hearts (A) and in hearts of in vivo treated rats (B). Phosphorylated and total Akt and AMPK in hearts perfused without (control) or with 1 μM MK2206, and with additional 25 μM DXR (C). Phosphorylated and total proteins of MAPK pathways (A and B) in isolated perfused rat hearts (D) and in hearts of in vivo-treated rats (E). Immunoblot analysis; total protein, tubulin, or GAPDH signals used for normalization. *P < 0.05, **P < 0.01 vs. control (n ≥ 3).
increased Akt signalling as observed by specific phosphorylation of Akt downstream targets, including GSK3 (Figures 4A and B, see also Supplementary material online, Figure S5). GSK3 is a prominent downstream effector of the DNA-PK–Akt pathway and essential to promote cell survival and inhibit apoptosis after DNA damage. Consistent with earlier studies, an increased activating phosphorylation of Akt itself was clearly detectable at Ser473 and Thr308 in the animal model, but below significance in perfused heart at both of these residues, despite strong phosphorylation of DNA-PK and GSK3. This may be due to multiple factors such as negative feedback loops by which activated downstream elements of the Akt-mTOR pathway limit upstream signalling, a particular time-course of Akt activation after DNA damage, or activation of local pools of Akt.

Finally, we directly tested the role of a putative Akt-AMPK cross-talk in DXR-induced AMPK inhibition by using pharmacological inhibition of Akt in perfused heart. MK2206, a specific allosteric Akt inhibitor, largely reduced Akt Ser173 phosphorylation, totally prevented Thr308 phosphorylation (Figure 4C), and inhibited phosphorylation of Akt substrates (see Supplementary material online, Figure S5). Importantly, MK2206 also abolished inhibition of AMPK in response to DXR, thus fully supporting an inhibitory Akt-AMPK cross-talk (Figure 4C), and reversed activation of TSC2, mTOR and other Akt substrates by DXR (see Supplementary material online, Figure S6). However, MK2206 did not normalize heart performance parameters, except in case of end-diastolic pressure (see Supplementary material online, Figure S1), likely because of pleiotropic deleterious effects of strong Akt inhibition. MK2206 did not inhibit DNA-PK and ERK, showing that it is specific at least in respect to these pathways (see Supplementary material online, Figure S7).

3.5 Doxorubicin activates MAPKs

Another kinase family reported to cross-talk with AMPK is MAPKs. MAPKs respond to a variety of stresses such as UV irradiation or reactive oxygen species in addition to growth factors and stretch stimuli. In particular, the extracellular signal-regulated protein kinase (ERK), a MAPK family member with pro-survival functions, is activated in the heart in response to practically every stress. In line with these characteristics and previous reports, an activation of ERK by DXR was observed in isolated heart (Figure 4D), and less so in the chronic model (Figure 4E). Pro-apoptotic c-Jun N-terminal kinase (JNK) was activated in both models, in particular in the chronic model, where also apoptotic markers were detectable (Figure 2J). Phosphorylation of the third prominent MAPK family member, p38, was less pronounced (Figure 4D).

3.6 Doxorubicin activates mTOR

mTOR integrates signals from the Akt, ERK, and AMPK pathways and plays an important role in regulating protein biosynthesis, cellular growth, and proliferation. Its activation by Akt and ERK is normally restricted by inhibitory AMPK signalling. As a reliable readout of in vivo mTOR activity, we quantified phosphorylation of the mTOR target p70S6K, which regulates mRNA translation. In both models, increased P-p70S6K revealed an activation of the mTOR pathway by DXR (Figure 5). Activation of mTOR was also analysed by its phosphorylation at Ser2448, which depends on active Akt, although its role for mTOR activity is not clearly established. In perfused heart, Ser2448 phosphorylation was increased at 25 μM DXR (Figure 5A). In hearts of treated animals (Figure 5B), phosphorylation of mTOR increased (not significant in the P-mTOR/mTOR ratio, but significant in the P-mTOR/GAPDH ratio; \( P < 0.01 \)), consistent with other reports on hearts of DXR-treated animals. Clearly, mTOR activation is expected in a situation where negative control via AMPK is blunted and positive control via Akt and ERK is reinforced. Finally, Akt activation of mTOR could occur via phosphorylation of TSC2 at Thr1462. In vivo DXR-induced phosphorylation of TSC2 at Thr1462 (Figure 5B). This phosphorylation was reversed under conditions of Akt inhibition (see Supplementary material online, Figure S6).
3.7 Compensatory mechanisms in the cardiac energetic network: up-regulation of BCK

Since AMPK is unable to fulﬁl its compensatory function in cellular energy homeostasis, we examined the status of another key factor for cardiac energy homeostasis, the creatine kinase energy buffer, and transfer system. Up-regulation of the cytosolic brain isoform of creatine kinase is one of the redundant regulatory and compensatory mechanisms observed in cardiac pathologies. Consistent with our previous ﬁndings, increased BCK protein was detectable in perfused heart, already at the low DXR concentration of 5 μM, and also in the animal model of chronic cardiotoxicity (see Supplementary material online, Figure S8).

4. Discussion

This study is a ﬁrst analysis of the LKB1–AMPK–mTOR signalling axis and its cross-talking signalling pathways as a potential mediator of DXR-induced cardiotoxicity. Two different models were chosen to represent acute and chronic toxicity: the DXR-perfused isolated rat heart and DXR-treated rats probed 4 weeks after the last injection. Both models mimic DXR plasma concentrations and myocardial alterations that are comparable to those in patients. The response of cell signalling to DXR was determined by the combined induction of at least three forms of stress: energetic, oxidative, and most of all genotoxic stress, as shown for the toxicity models in this and other studies. We show that under these conditions, the fuel-sensing AMPK signalling is not activated but instead paradoxically suppressed, whereas survival signalling by DNA-PK–Akt–GSK3, as well as stress signalling by MAPK, is up-regulated. As a consequence, mTOR, the main regulator of protein synthesis and cell growth, which integrates signals from the several protein kinase pathways, becomes activated. This signalling pattern contains some cytoprotective elements, such as pro-survival Akt–GSK3 signalling triggered by DNA damage via DNA-PK, which counteracts apoptosis due to genotoxic stress, or transient anti-apoptotic ERK activation. However, there are several elements that likely contribute to cardiotoxicity. First, inhibition of compensating pleiotropic AMPK downstream signalling exacerbates energy stress. This will limit the capacity for ATP regeneration, e.g. via fatty acid oxidation, and maintain or reinforce numerous energy-consuming biosynthetic processes, like via the relieved inhibition of mTOR. This will also make cardiomyocytes less resistant to additional physiological and pathological stressors such as strenuous exercise, hypoxia, or ischaemia.

DXR induces not only energetic imbalance, a direct AMPK activator, but also reactive oxygen and nitrogen species and genotoxic stress, which should stimulate AMPK activity by more indirect mechanisms. In both analysed models, at least two of these forms of DXR-induced stress are present at a level that activates AMPK in different cellular systems. The lack of AMPK activation by DXR and even more the diminished basal phosphorylation by DXR is therefore unexpected. However, it is consistent with some other reported DXR effects: (i) a change in the proﬁle of cardiac energy substrate utilization with a decrease in oxidation of both fatty acids and glucose, (ii) reduced exercise tolerance affecting up to 70% of cancer patients during and after therapy, (iii) protection against DXR cardiotoxicity by compounds and regimens activating AMPK such as resveratrol, adiponectin, caloric restriction, and physical exercise, and aggravation by conditions suppressing AMPK signalling such as obesity and high fat diet.

The cardiac metabolic network is characterized by an unusual stability. Energy homeostasis is maintained by multiple regulatory mechanisms, including allosteric regulations and feedback loops, micro-compartmentation, or metabolic channelling. Compensatory mechanisms within the cardiac energy network occur early under DXR treatment and include, for example, up-regulation of brain-type creatine kinase and increased expression of other transcripts involved in ATP generation. AMPK activation in the heart, in contrast to most other tissues, seems rather to act as a last safeguard during severe energy deprivation and pathological situations, such as cardiac ischaemia. Thus, DXR-induced inhibition of AMPK is expected to generate a negative feedback loop that could not only contribute to the aetiology of the pathological phenotype, but also aggravate it in advanced stages of DXR-induced cardiomyopathy or during additional energy stress as observed with patients.

In search of mechanisms responsible for AMPK inhibition, we have analysed most known pathways upstream of or cross-talking with AMPK. The by far most important AMPK kinase in the heart, the tumour suppressor LKB1 which is considered to be constitutively active, was not affected in terms of the expression level. It even showed increased deacetylation and phosphorylation, reported to favour cytosolic localization and downstream signalling. Protein adducts with HNE, a byproduct of lipid peroxidation reported to inhibit LKB1–AMPK signalling in the heart upon oxidative insult, were neither found increased. Levels of the second AMPK kinase, CAMKKβ, were also unchanged. Phosphatases regulating AMPK activity are less deﬁned, but at least PP2C activity was not altered. Thus, no evidence for inhibited AMPK upstream signalling could be found, although we cannot deﬁnitely reject decreased activities of LKB1 and CamKKβ due to other, unknown mechanisms. More likely, inhibitory cross-talk with other signalling pathways can play an important role. Akt, and as suggested more recently also ERK, can cross-talk with and inhibit AMPK, and to a decreasing activity in advanced stages of DXR-induced cardiomyopathy, both pathways were strongly activated by DXR in both examined models.

The interplay of AMPK with Akt is especially pronounced in the heart. We show here that DNA-PK, recently located upstream of Akt, represents an important and novel element in the cardiac response to DXR. DNA-PK, a crucial component of the DNA repair machinery for double-strand breaks, shows rapid and strong phosphorylation and activation by DXR. It thus connects DNA-damage signalling to activation of Akt, inhibition of AMPK, and even more pronounced phosphorylation of further downstream elements such as GSK3 that promote cell survival after genotoxic stress. The negative Akt–AMPK cross-talk is conﬁrmed in our perfused heart model by pharmacological inhibition of Akt which reverts AMPK inhibition and downstream mTOR activation. While activation of the DNA-PK–Akt–GSK3 pathway was comparable in both models examined here, apoptosis was lower in perfused heart when compared with the chronic model, correlating with the activation pattern of MAPKs. While phosphorylation of pro-survival ERK was prominent in the former model, pro-apoptotic JNK became activated in the latter, in line with previous reports of us and others. Apoptosis, for which cardiomyocytes are relatively resistant, heavily contributes to heart failure when occurring. Pathways counteracting apoptosis, as DNA-PK–Akt–GSK3 and ERK, may be crucial to preserve cardiomyocyte viability during genotoxic stress. In the DXR-challenged heart, the up-regulation of Akt confers signiﬁcant protection.
Combined inhibition of AMPK and activation of Akt and ERK by DXR will lead to elevated mTOR signalling, as we observed by increased phosphorylation of mTOR (at Ser2448) and its downstream target p70S6K. This will stimulate energetically costly biosynthetic pathways in protein biosynthesis and cell growth, thus generally targeting p70S6K. This will stimulate energetically costly biosynthetic pathways in protein biosynthesis and cell growth, thus generally enhancing DXR-induced energy deficits, but also generating growth-stimulating signals. Red arrows indicate up- or down-regulation by DXR; red crosses indicate inhibition of signalling.

**Figure 6** Doxorubicin-induced combined energetic, genotoxic, and oxidative stress inhibits AMPK. AMPK inhibition is at least partially due to the regulatory cross-talk with Akt and MAPK pathways, largely triggered by DNA damage signalling via DNA-PK. AMPK inhibition and related activation of mTOR (i) initiate a negative feedback that aggravates DXR-induced energy deficits and makes heart less resistant to additional energy challenge and also (ii) generate growth-stimulating signals. Depending on the context, mTOR activation can enhancing DXR-induced energy deficits, but also generating growth-stimulating pathways in protein biosynthesis and cell growth, thus generally enhancing DXR-induced energy deficits, but also generating growth-stimulating signals.

In summary, in the DXR-challenged heart, combined energetic, oxidative and genotoxic stress elicits a specific response where AMPK is paradoxically inhibited at least partially by the known regulatory cross-talk with activated Akt and MAPK pathways, largely triggered by DNA damage signalling via DNA-PK (Figure 6). This occurs in the acute toxicity model, as well as in the more chronic model probed 4 weeks after the last DXR injection. Although activation of DNA-PK—Akt and MAPK pathways has some protective consequences, impaired energy signalling by AMPK will initiate a negative feedback loop that potentiates DXR-induced energy deficits and seriously compromises heart resistance to additional energy stress. Activation of growth-stimulating mTOR by combined AMPK inactivation and Akt/ERK activation will also contribute to the pathological cardiac phenotype in chronic DXR toxicity.

**Supplementary material**

Supplementary material is available at Cardiovascular Research online.

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