Defining the action spectrum of potential PGC-1α activators on a mitochondrial and cellular level in vivo

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Received September 30, 2013; Revised and Accepted December 9, 2013

Previous studies have demonstrated a therapeutic benefit of pharmaceutical PGC-1α activation in cellular and murine model of disorders linked to mitochondrial dysfunction. While in some cases, this effect seems to be clearly associated with boosting of mitochondrial function, additional alterations as well as tissue- and cell-type-specific effects might play an important role. We initiated a comprehensive analysis of the effects of potential PGC-1α-activating drugs and pharmaceutically targeted the PPAR (bezafibrate, rosiglitazone), AMPK (AICAR, metformin) and Sirt1 (resveratrol) pathways in HeLa cells, neuronal cells and PGC-1α-deficient MEFs to get insight into cell type specificity and PGC-1α dependence of their working action. We used bezafibrate as a model drug to assess the effect on a tissue-specific level in a murine model. Not all analyzed drugs activate the PGC pathway or alter mitochondrial protein levels. However, they all affect supramolecular assembly of OXPHOS complexes and OXPHOS protein stability. In addition, a clear drug- and cell-type-specific influence on several cellular stress pathways as well as on post-translational modifications could be demonstrated, which might be relevant to fully understand the action of the analyzed drugs in the disease state. Importantly, the effect on the activation of mitochondrial biogenesis and stress response program upon drug treatment is PGC-1α dependent in MEFs demonstrating not only the pleiotropic effects of this molecule but points also to the working mechanism of the analyzed drugs. The definition of the action spectrum of the different drugs forms the basis for a defect-specific compensation strategy and a future personalized therapeutic approach.

INTRODUCTION

Mitochondria play an important role in many degenerative diseases, either by being directly involved in the disease pathology or through indirect mechanisms and secondary effects (1–3). Mitochondrial function is reciprocally linked to many cellular degenerative processes and stress programs such as inflammation and starvation signaling (4,5). A finely tuned and complex molecular system likely links mitochondrial processes to cellular stress-responsive pathways to ensure bioenergetic homeostasis in a cell- and probably tissue-specific way.

Potential players might be the PGC factors, a family of transcriptional co-activators with PGC-1α being the most studied member (4,5). PGC-1α is not only an important regulator of the mitochondrial biogenesis program but is also involved in the regulation of several stress programs and might thereby integrate mitochondrial biogenesis into cellular stress adaption (4,5). PGC-1α can be activated via the PPAR, AMPK or Sirt1 pathway, either through increased expression (PPAR, AMPK) or through post-translational modification (PTMs; AMPK, Sirt1) (6–8). While PGC-1α is not a direct drug target yet, several substances and drugs that can activate PPAR, AMPK and Sirt1 are established. These could therefore indirectly activate PGC-1α and its down-stream targets (8). Several groups, including our own, could previously show that activation of the PGC-1α pathway has a potential therapeutic benefit in muscular and neurodegenerative disorders associated with mitochondrial dysfunction in cellular and murine models (9–22). The therapeutic benefit of the transgenic PGC-1α activation can in some
cases at least be partially recapitulated by drug stimulation of PGC-1α activation (9,15,19). In some cases, the administration of the respective drug had a clear boosting effect on mitochondrial processes such as oxidative phosphorylation (OXPHOS) or fatty acid oxidation (9,11,12,15,19,23), while in other cases no significant effect could be observed (9,11,15). Some studies report a strong tissue specificity of the observed effects (9,11,22). Interestingly, some studies revealed that the administration of drugs, which influence the PGC-1α pathway, modulate cellular degenerative processes and could thereby influence disease progression via non-bioenergetic and maybe PGC-independent pathways in addition to the impact on mitochondria (11,12,23).

Several drugs or pathway-activating pharmaceuticals have been postulated to enhance the PGC-pathway or mitochondrial biogenesis. In the present study, we aimed to expand on these results and to define the action spectrum of different postulated PGC-1α activating drugs: we assessed their actual activation capacity on the PGC-pathway itself. With regard to mitochondria, we not only analyzed changes in OXPHOS or mitochondrial mass, which are the expected consequences of PGC-1α activation, but also supramolecular assembly and stability of OXPHOS proteins, which have not yet been connected to the PGC-1α pathway. This approach lead to a comprehensive understanding on how these drugs could affect mitochondria, irrespective of their PGC-activating potential. We also assessed the impact on cellular stress response programs, including starvation and inflammatory signaling, which have been implicated in degenerative diseases. In addition, we analyzed the impact on PTMs, including acetylation and phosphorylation. Since PTMs are increasingly recognized as a major factor in stress adaptation and enzyme regulation (24–27).

In this study, we pharmaceutically targeted the PPAR (bezafibrate, rosiglitazone), AMPK (AICAR, metformin) and Sirt1 pathways (resveratrol) in HeLa cells as well as in differentiated neuronal cells to get insight into cell type specificity. In addition, we took advantage of PGC-1α-deficient mouse embryonic fibroblasts to determine the role of PGC-1α in the action spectrum of the analyzed drugs. To understand tissue-dependent effects, we used bezafibrate as a model drug to assess the effect on an organ-specific level in a murine model. Our results indicate that drugs activate the PGC pathway and alter mitochondrial protein levels as well as cellular stress pathways in a strong cell-type- and dose-dependent manner. Moreover, in MEFs, we could demonstrate that the observed changes are dependent on PGC-1α. Intriguingly, we can show that all drugs affect supramolecular assembly of OXPHOS complexes as well as OXPHOS protein stability. These findings hint at a novel feature of mitochondrial function being regulated by the assessed drugs.

RESULTS

Pharmaceuticals affect the activation of the PGC pathway in a drug-specific way

Bezafibrate, rosiglitazone, AICAR, metformin and resveratrol have been postulated to activate the PGC pathway and thereby indirectly affect mitochondrial biogenesis and function. However, this activation potential has only been demonstrated for bezafibrate and AICAR in patient fibroblasts and in selected murine tissues so far (9,12,15,19,21–23,28). To understand the impact of the individual pharmaceuticals on activation of the PGC-signaling pathway, we cultivated HeLa cells in the presence of increasing concentrations of bezafibrate, rosiglitazone, AICAR, metformin and resveratrol for 72 h and analyzed the steady-state levels of PGC-1α and PGC-1β, which are both implicated in the mitochondrial biogenesis pathway. Since PGC-1α activity is not only controlled by increased protein levels but also through PTMs, we also assessed the steady-state levels of the nuclear respiratory factors NRF1 and NRF2 for general changes in the mitochondrial biogenesis program.

The PPAR agonist bezafibrate significantly increased PGC-1α steady-state levels, even at the lowest concentration of 150 μM. At bezafibrate concentrations of 400 μM and above, increased PGC-1β protein levels were observed. NRF1 and NRF2 protein levels did not significantly increase in response to bezafibrate treatment. TFAM protein levels were up-regulated, indicating activation of the PGC pathway (Fig. 1A and Supplementary Material, Fig. S1A). This result is similar to previous findings in patient fibroblast and cybrid cell lines (21).

The second PPAR agonist we tested was rosiglitazone. A robust and significant increase of both PGC-1α and PGC-1β protein levels was observed. NRF1 protein levels remained unchanged, whereas NRF2 levels increased at concentrations >45 μM. We also observed a significant up-regulation of TFAM steady-state levels, which was lost at the highest concentration compared with the vehicle-treated control (Fig. 1B and Supplementary Material, Fig. S1A).

Upon treatment with 1 and 2 mM of the AMPK-activator AICAR PGC-1α levels increased. PGC-1β levels were increased as well. However, we observed strong variations when analyzing the effects of AICAR on PGC levels. Therefore, not in all points statistical significance could be reached. NRF1 levels were found to be increased at concentrations <1 mM AICAR when compared with the vehicle-treated control, while NRF2 steady-state levels did not respond to AICAR treatment. TFAM protein levels were increased at all concentrations (Fig. 1C and Supplementary Material, Fig. S1A). This result is in accordance with a previous report where TFAM expression is increased in response to AICAR treatment (15).

Surprisingly, we did not observe gross changes after treatment with the AMPK-activator metformin (Fig. 1D and Supplementary Material, Fig. S1A).

Growth of HeLa cells in the presence of resveratrol resulted in slightly, but significantly increased PGC-1α protein levels at low concentrations (up to 75 μM) which dropped to control levels at higher concentrations. PGC-1β protein levels increased with increasing concentration of resveratrol. NRF1 and NRF2 did not change significantly in response to the drug. TFAM protein levels were increased at the lowest concentration of resveratrol (50 μM) and dropped to control levels at higher concentrations (Fig. 1E and Supplementary Material, Fig. S1A).

Mitochondrial and OXPHOS protein markers are increased in response to drug treatment

We next analyzed the effect of the different drugs on markers of OXPHOS enzymes and on mitochondrial mass in HeLa cells.
Bezafibrate treatment of HeLa cells resulted in an increase of the steady-state levels of NDUFB8 of complex I, the FeS subunit of complex II and UQCR2 of complex III. VDAC (outer membrane marker) and TIM23 of the inner mitochondrial import machinery (inner membrane marker) also showed significantly increased protein levels. Instead no significant change for...
the matrix-localized Polrmt and OXPHOS complex V subunit ATPα could be observed (Fig. 1A and Supplementary Material, Fig. S1B).

Rosiglitazone, which induced a robust up-regulation of PGC-1α/β and TFAM protein levels, lead to a significant increase in all analyzed markers for OXPHOS subunits, markers for mitochondrial mass (TIM23, VDAC) and matrix proteins (Polrmt, Gfm1) (Fig. 1B and Supplementary Material, Fig. S1B).

While metformin had no obvious effect on the levels of any of the analyzed markers (Fig. 1D and Supplementary Material, Fig. S1B), AICAR treatment resulted in increased protein levels of several OXPHOS subunits (ATP5α, UQCR2) and mitochondrial matrix proteins (Polrmt, Gfm1). Other OXPHOS markers and markers for mitochondrial mass did not significantly change in response to the treatment (Fig. 1C and Supplementary Material, Fig. S1B).

Treatment with resveratrol had ambiguous effects on the analyzed markers with increasing drug concentration: at concentrations < 75 μM, we observed robust and significant up-regulation of UQCR2, NDUF8 and FeS subunit. At concentrations of 100 μM and above, all markers were significantly decreased compared with the vehicle-treated control (Fig. 1E and Supplementary Material, Fig. S1B).

All analyzed drugs increase the levels of OXPHOS supercomplexes

As described above, we only see a slight effect on mitochondrial protein levels in response to the drug treatment in HeLa cells independent of the degree of PGC-1α pathway activation. We next asked the question, whether the drugs could affect OXPHOS on other levels than modulation of mitochondrial protein levels. We therefore analyzed the impact of the drug treatment on the steady-state levels of fully assembled OXPHOS complexes and their supramolecular assembly. To test for OXPHOS complexes, we performed Blue-Native (BN)-PAGE analysis of laurylmaltoside-solubilisates, where the individual complexes can be resolved. Except for metformin, we did not observe a major effect of the analyzed drugs on the levels of OXPHOS complex III and IV, which is in line with the only mild effect of the drugs on OXPHOS subunit protein level. In the case of metformin, a mild increase in complex IV was observed. Complex II steady-state levels were only affected in rosiglitazone-treated cells, where an increase was observed. For all other drugs, no major change in complex II levels was detected (Supplementary Material, Fig. S2A). Free complex I could not be resolved with this experimental setup in our hands.

Next, we analyzed supramolecular assemblies of the OXPHOS enzymes by performing a BN-PAGE analysis of digitonin extracts. Remarkably, we observed a major increase in supercomplexes for all tested drugs as well as major increases in the assembly of complex III and IV (Fig. 2A). Only in rosiglitazone-treated samples, this increase in supramolecular complexes was associated with an increase in complex II (Fig. 2A), which was also observed in laurylmaltoside-extracted cells (Supplementary Material, Fig. S2A). Since we did not observe an increase in the individual complexes, this increase in supercomplexes in response to drug treatment might involve assembly and stability of the supercomplexes itself. When we quantified the amount of available complex III, that is present in the free pool or in supramolecular assemblies, we determined that in control cells, ~80% of complex III is present in the free form. Upon drug treatment, complex III partitioning shifted from less free complex III to more CIII + CIV supercomplex assemblies and the percentage of free complex III in treated cells ranges from ~55 to 70% of total complex III. This finding indicates a favorable environment of supramolecular assemblies of OXPHOS complexes in response to drug treatment (Fig. 2B).

Metformin and resveratrol treatment affect mitochondrial morphology

Changes in mitochondrial morphology are an important factor in controlling and maintaining mitochondrial function (29) and have been recently reported to affect supercomplex assembly (30). To determine if and how the drugs affect mitochondrial morphology and could thereby indirectly contribute to the above observed alteration, we assessed mitochondrial shape by immunostaining of the outer mitochondrial membrane protein Tom20 in treated HeLa cells. (Supplementary Material, Fig. S3). For simple quantification, we chose to categorize mitochondria components into three classes (small, medium, large) and calculated percentage of components as well as the area occupied by the components (Supplementary Material, Fig. S3A, see also Methods).

With our approach, no significant change in mitochondrial area distribution is evident in bezafibrate, rosiglitazone and AICAR treatments (Supplementary Material, Fig. S3A and B). Treatment with metformin indicates an elongation of mitochondria, as the quantification shows a significant increase in medium mitochondria class contribution to the overall mitochondria area (Supplementary Material, Fig. S3A and B). A shift towards elongated mitochondria has been recently reported in metformin-treated diabetic rats (31). Resveratrol had the opposite effect; a significant shift towards smaller mitochondria was detected (Supplementary Material, Fig. S3A and B). Interestingly, PPAR agonists, two of which (bezafibrate and rosiglitazone) failed to induce a change in mitochondrial morphology here in basal settings, recovered mitochondrial dynamics in H2O2-challenged cells implying that certain drugs might exert their function predominantly under cellular stress conditions (32).

OXPHOS complexes have increased stability upon drug treatment

We next tested whether drug treatment impacts the stability and turnover of OXPHOS complexes. We therefore performed metabolic pulse-chase labeling of mitochondrial encoded proteins and followed the stability of the assembled OXPHOS complexes via BN-PAGE. Densiometric analysis revealed a lower turnover of complex IV in response to treatment with bezafibrate, rosiglitazone and resveratrol, while AICAR and metformin showed only weak effects (Fig. 2C). All analyzed drugs had a stabilizing effect on complex V as evident from the slower decay of the labeled complex signal (Supplementary Material, Fig. S2B) with bezafibrate and metformin showing the strongest effects. The signals for complex III and supercomplexes were too weak for densiometric analysis and turnover quantification.
Drug treatment alters cellular acetylation and phosphorylation state

After having assessed the impact of the drugs on mitochondrial processes, we now wondered if the drugs could interfere with other cellular pathways that might be implicated in cellular function other than mitochondria. PTMs, which can modulate protein function, and stability are increasingly recognized to contribute to the regulation of cellular pathways (24–27). Prominent PTMs include lysine acetylation and phosphorylation, which are both linked to changes in protein activity and stability. We analyzed the effects on PTMs in cellular extracts in responses to drug treatments by assessing overall lysine acetylation as well as phosphorylation of kinase substrates (PKA, MAPK and PKC) using western blotting and subsequent densiometric analysis.

When probing for overall lysine acetylation, we did not observe a major effect of bezafibrate on this class of PTM. Rosiglitazone significantly increased lysine acetylation at low concentrations, but this effect was lost at higher concentrations. Low concentration of AICAR (up to 0.5 mM) resulted in increased cellular protein acetylation, which decreased at higher concentrations. Metformin treatment resulted in increased protein acetylation with increasing drug concentration. Remarkably, treatment with resveratrol decreased the cellular acetylation, which is in accordance with the stimulation of the deacetylase Sirt1 (Fig. 3A).

Bezafibrate treatment resulted in increased phosphorylation of PKA-substrates, while no significant effect on phosphorylation of PKC and MAPK substrates was observed in the analyzed concentration range. Rosiglitazone increased the phosphorylation of PKA and PKC substrates, while no significant effect on MAPK substrates was detected. AICAR treatment showed ambiguous effects: at all analyzed concentrations, MAPK-mediated phosphorylation was significantly decreased, while phosphorylation of PKA and PKC substrates was increased at concentrations of up to 1 mM. Metformin lead to a general decrease in the phosphorylation of all analyzed substrates. Resveratrol treatment resulted in decreased phosphorylation of PKC substrates, while MAPK substrates were significantly more phosphorylated at concentrations of up to 100 μM. No major effects on PKA substrates were observed (Fig. 3B).

Further work is necessary to fully understand the molecular basis and implications of these findings, especially the impact on important cellular signaling pathways in response to drug treatments.

Analyzed drugs affect cellular stress pathways in a drug-specific manner

Next, we analyzed the impact of drug treatments on cellular signaling pathways involved in the adaption of cells to challenges and degenerative processes in the disease state. Key players in cellular stress adaption, such as mTOR, AKT and AMPK activation were analyzed (33,34). A decrease in the inflammatory response under neurodegenerative conditions was observed in response to bezafibrate and could therefore be a possible contribution to the therapeutic potential of bezafibrate and other drugs (23). Therefore, we analyzed changes in inflammatory markers NFκB, IκB and COX2.

This analysis revealed striking differences between applied drugs on the activation of these molecules: bezafibrate- and AICAR-induced dephosphorylation of mTOR, while metformin and resveratrol had no significant effect, which was also reflected in unchanged mTOR-substrate phosphorylation (Fig. 4A and C).
and Supplementary Material, Fig. S4A and B). Despite the decreased levels of pmTOR/mTOR, mTOR-substrate phosphorylation remained unchanged in bezafibrate-treated cells. AICAR instead treatment resulted in decreased mTOR-substrate phosphorylation. Rosiglitazone treatment caused increased mTOR phosphorylation at 15 \( \mu \text{M} \) which decreased at higher concentrations below control levels (Fig. 4B and Supplementary Material, Fig. S4A), which is also reflected by mTOR substrate phosphorylation (Supplementary Material, Fig. S4B).

Bezafibrate slightly decreased AMPK activation (Fig. 4A and Supplementary Material, Fig. S4A), but did not result in gross changes in AMPK-substrate phosphorylation (Supplementary Material, Fig. S4B). Rosiglitazone treatment increased AMPK phosphorylation (Fig. 4B and Supplementary Material, Fig. S4A) and this activation is also visible in increased phosphorylation of AMPK substrates (Supplementary Material, Fig. S4B). AICAR, an AMPK activator, increases AMPK activation at doses up to 1 \( \text{mM} \), indicated by an increased pAMPK/AMPK ratio and increased AMPK-substrate phosphorylation (Fig. 4C, Supplementary Material, Fig. S4A and B). Metformin, the second analyzed AMPK activator, surprisingly decreased phosphorylated versus unphosphorylated NFkB, which is lost at higher concentrations. Metformin and resveratrol also decreased the ratio of phosphorylated NFkB versus total NFkB as well as IkB levels at higher concentrations. On the other hand, resveratrol increased COX2 levels with increasing concentrations. Bezafibrate had no gross effect on the analyzed inflammatory markers in HeLa cells (Fig. 4 and Supplementary Material, Fig. S4A).

Changes in general stress–response markers, such as HO-1, Hsp70 and SOD2, were tested. Hsp70 was not affected for any analyzed drug with the exception of the highest concentration of resveratrol (150 \( \mu \text{M} \)), where Hsp70 levels were drastically increased (Fig. 4 and Supplementary Material, Fig. S4A).

**Inflammatory signaling and stress markers are impacted by drug treatment**

Markers of inflammatory signaling were also responsive to drug treatment. Rosiglitazone and AICAR significantly increased the protein levels of COX2 and IkB. At low concentrations, rosiglitazone also increased the relative amount of phosphorylated versus unphosphorylated NFkB, which is lost at higher concentrations. Metformin and resveratrol also decreased the ratio of phosphorylated NFkB versus total NFkB as well as IkB levels at higher concentrations. On the other hand, resveratrol increased COX2 levels with increasing concentrations. Bezafibrate had no gross effect on the analyzed inflammatory markers in HeLa cells (Fig. 4 and Supplementary Material, Fig. S4A).

Changes in general stress–response markers, such as HO-1, Hsp70 and SOD2, were tested. Hsp70 was not affected for any analyzed drug with the exception of the highest concentration of resveratrol (150 \( \mu \text{M} \)), where Hsp70 levels were drastically increased (Fig. 4 and Supplementary Material, Fig. S4A).

HO-1 levels were significantly decreased in response to rosiglitazone, AICAR and resveratrol treatment, while metformin did not significantly alter HO-1 protein levels. Bezafibrate influenced HO-1 in a concentration dependent manner leading to a
significant decrease at higher drug concentrations (Fig. 4 and Supplementary Material, Fig. S4A).

SOD2 protein levels were significantly increased in response to all analyzed drugs except for rosiglitazone, which did not change SOD2 levels (Fig. 4 and Supplementary Material, Fig. S4A).

Resveratrol exerts cell toxicity at high concentrations

Since we had observed strong changes of some markers at high drug concentrations, we wondered whether these high concentrations would affect cell viability. We therefore measured the number of live and dead cells after treatment. Bezafibrate,
rosglitazone, AICAR and metformin did not significantly affect cell number and cell viability as judged from >93% live cells at time of harvest (Supplementary Material, Fig. S5). However, resveratrol decreased live cell numbers and the percentage of life cells at the end of treatment at 100 μM and above. Decreased cell numbers were also observed for the highest bezafibrate and AICAR concentration; however, no increase in dead cells was detected (Supplementary Material, Fig. S5).

**Pharmaceuticals affect neuronal cells in a different manner than HeLa cells**

After describing the action spectrum of the investigated drugs in HeLa cells (see Table 1 for summary), we chose to analyze drug-specific effects in a second cell system (differentiated neuronal SH-SY5Y cells) to test whether the described characteristics could be generalized.

When we checked for activation of the PGC-signaling pathway, we observed that bezafibrate treatment significantly increased PGC-1α and TFAM levels in neuronal cells (as we have reported in a previous study (23)), while no gross effect on PGC-1β protein and NRF1 protein levels was observed (Fig. 5A and Supplementary Material, Fig. S6A). Rosiglitazone reacted in the same manner (Fig. 5B and Supplementary Material, Fig. S6A).

AICAR treatment resulted in a slight increase of PGC-1α protein levels, associated with an increase in TFAM protein levels for concentrations up to 1 mM. At 1 mM and above, we observed a striking/strong increase in PGC-1β, while TFAM activation was lost. No gross alterations in NRF1 levels were observed (Fig. 5C and Supplementary Material, Fig. S6A). Metformin did not show strong effects on PGC-1α and NRF1 levels, but increased PGC-1β and TFAM levels significantly (Fig. 5D and Supplementary Material, Fig. S6A). AICAR and Metformin treatment increased phosphorylation of AMPK substrates, indicating that we were working in a functional concentration range in neuronal cells (Supplementary Material, Fig. S6C).

Resveratrol had to be used at lower concentrations than in HeLa cells: in our neuronal cell model, only concentrations up to 50 μM resveratrol were tolerated. Below 50 μM, resveratrol increased PGC-1α levels, but no gross effects on PGC-1β or TFAM was detected (Fig. 5E and Supplementary Material, Fig. S6A). We were not able to detect NRF2 in any setup in neuronal cells.

Mitochondrial markers were responsive to the drug treatment as well: bezafibrate treatment resulted in a general increase of mitochondrial markers in neuronal cells, while rosiglitazone had ambiguous effects with a decrease in the complex I marker NDUFB8 and an increase in TIM23 (Fig. 5A and B and Supplementary Material, Fig. S6A). AICAR and metformin treatment increased mitochondrial protein levels at low concentrations (up to 1 mM) while higher concentrations resulted in a decrease of the steady-state levels (Fig. 5C and D and Supplementary Material, Fig. S6A). Low concentrations of resveratrol (below 50 μM) increased OXPHOS markers while leaving TIM23 unaffected (Fig. 5E and Supplementary Material, Fig. S6A).

A marked difference in the response of cellular stress markers upon drug treatment was observed in neuronal cells compared with HeLa cells. Bezafibrate, AICAR and resveratrol significantly increased mTOR phosphorylation, while no effect was
Figure 5. Impact of drugs on the PGC-1α signal pathway, OXPHOS and stress response in neuronal cells. Differentiated SH-SY5Y cells were treated for 120 h with the indicated concentration of drugs in the media. Activation of the PGC-signaling pathways was assessed by measuring steady-state levels of PGC-1α as well as PGC-1α targets NRF1, NRF2 and TFAM. To assess the activation of mitochondrial biogenesis and mitochondrial mass, the level of different mitochondrial proteins including OXPHOS subunits (FeS (complex II), NDUFB8 (complex I)), outer mitochondrial membrane protein (VDAC) and a subunit of mitochondrial import machinery (TIMM23) were analyzed. Markers of cellular signaling pathways (mTOR, AKT, AMPK), inflammation (NFKB, IKB and COX2) and general stress markers (HO-1, SOD2, Hsp70) were assessed. Shown are quantifications of western blot analysis (see Supplementary Material, Fig. S6A for representative blots) by densitometry. ∗P < 0.05; ∗∗P < 0.01; ∗∗∗P < 0.001.
observed for rosiglitazone. High concentrations of metformin even decreased mTOR activation. Resveratrol increased AMPK activation, while no clear effect on the pAMPK/AMPK ratio could be detected in all other scenarios. Phosphorylation of AKT with respect to total AKT was increased for all analyzed drugs (Fig. 5 and Supplementary Material, Fig. S6B).

With respect to inflammatory signaling, drug-specific effects were observed as well: bezafibrate and metformin increased the analyzed inflammatory markers pNFκB/NFκB, IkB and COX2, while rosiglitazone had the opposite effect. AICAR increased the relative amount of pNFκB versus the non-phosphorylated form, while IkB and COX2 were decreased. Resveratrol had no gross effect on the analyzed markers (Fig. 5 and Supplementary Material, Fig. S6B).

With respect to general stress markers, bezafibrate and rosiglitazone did increase HO-1 levels, while AICAR and metformin had no effect. Resveratrol decreased HO-1 levels. SOD2 protein levels were significantly increased in response to bezafibrate, AICAR, metformin and low concentrations of resveratrol, while rosiglitazone had no major effect. Hsp70 protein levels followed a similar pattern with a slight, but significant increase in response to bezafibrate, AICAR, metformin and low concentrations of resveratrol (Fig. 5 and Supplementary Material, Fig. S6B).

The observation from HeLa cells that high concentrations of resveratrol result in an increasing amount of dead cells after the treatment regimen holds also true for neuronal cells. (Supplementary Material, Fig. S7). In addition, high drug concentrations decreased cell numbers while cell viability was not affected which is consistent with a reduced growth rate (Supplementary Material, Fig. S9). The reduced growth rate is also observed in different cell types after bezafibrate treatment, possibly caused by a shift to a more aerobic phenotype (21). It is plausible that a similar scenario occurs in our setting since we cannot detect cell toxicity (as judged by unaltered cell viability) but an increase in mitochondrial metabolism markers.

Similar to our observations in HeLa cells, metformin and resveratrol had a slight, but significant effect on mitochondrial morphology in differentiated neuronal cells. Resveratrol showed decreased medium size and increased small sized mitochondria, while metformin decreased the number of large-sized mitochondria. None of the drugs affected the occupancy of the mitochondrial area (Supplementary Material, Fig. S8).

Bezafibrate affects the PGC pathway and cellular stress programs in a tissue-specific manner

Our results in different cell lines indicate that the analyzed drugs have a cell-type-specific effect on PGC-1α activation, mitochondrial markers as well as on cellular stress programs. We have reported before that bezafibrate injection had only a minor effect on brain mitochondrial markers, whereas liver mitochondrial markers were up-regulated (23). We therefore tested whether other tissue-specific effects could be found, expanding this line of research. We therefore used bezafibrate as a model drug to investigate whether pharmaceutical activation of the PGC-1-pathway underlies tissue-specific constraints.

As described before, we did not observe any significant change in mitochondrial markers in the brain upon bezafibrate injection. The same is true for heart tissue, where mitochondrial markers did not change in response to the drug. Here, PGC-1α protein levels even decreased in response to the treatment (Fig. 6A and Supplementary Material, Fig. S9A). In liver, we observed a mild, but significant increase in the mitochondrial markers SDHA and Tim23 along with an increase in PGC-1α, NRF1 and TFAM protein levels. Interestingly, the mitochondrial markers in skeletal muscle remained unaffected by 7 days bezafibrate injection. However, a strong increase in PGC-1α protein levels as well as a small but significant increase in TFAM protein levels was observed (Fig. 6A and Supplementary Material, Fig. S9A). A possible explanation of these observations is that the 7-day treatment is too short to induce gross mitochondrial biogenesis and that a longer time frame would be necessary to observe protein level changes.

The analysis of cellular stress–response markers showed the strongest effects of bezafibrate treatment. In the brain, we observed a marked increase in COX2 and a mild increased in IkB. This finding is in agreement with one of our previous results in a mouse model, where bezafibrate could decrease the inflammatory response in the brain (23). In addition, bezafibrate injection resulted in an increase of the stress markers HO-1 and Hsp70 in the brain. These two proteins were also markedly increased in liver of injected mice. In liver, an increase in pAMPK/AMPK ratio indicated a specific activation of AMPK. This activation was also seen in heart, along with increased pAKT levels. Increased phosphorylation of AKT substrates was observed, which indicates an activation of the AKT-signaling pathway. In contrast to liver and brain, bezafibrate injection resulted in a specific and marked decrease of IkB in the heart in injected mice. This specific decrease in the IKB inflammatory marker was also seen in skeletal muscle and was accompanied by a dramatic increase of Hsp70 (Fig. 6).

Furthermore, a tissue-specific effect of bezafibrate injection on protein acetylation was found. While our results in cell lines showed that bezafibrate did not induce alterations of total cellular lysine acetylation, we found a strong increase in the total protein acetylation state in liver and skeletal muscle tissue after bezafibrate injection in mice but not in brain and heart tissue. This intriguing finding strongly implies a tissue-specific effect of bezafibrate on inflammatory signaling, starvation signaling and PTMs and demonstrates how the effect of drug treatment can diverge when comparing cellular and mouse models (Supplementary Material, Fig. S9B, Tables 1 and 2).

Effects on mitochondrial biogenesis and stress response are PGC-1α dependent in MEFs

To assess which of the effects described above are PGC-1α dependent, we used WT and PGC-1α KO immortalized mouse embryonic fibroblast (35) and subjected them to the same drug treatment as HeLa and differentiated SH-SY5Y cells. AICAR and resveratrol had a strong toxic effect in the MEF system making an analysis impossible. We therefore focused on the analysis of bezafibrate, rosiglitazone and metformin, where administration did not compromise cell growth. As before, we assessed in both WT and PGC-1α KO MEFs markers of mitochondrial biogenesis and OXPHOS as well as several cellular stress markers including inflammatory and starvation response along with general stress markers.
In all cases, we observed in WT-MEFs robust activation of the mitochondrial biogenesis program with increasing drug concentrations as indicated by increased protein levels of the PGC-1α pathway marker protein TFAM and different mitochondrial proteins (Fig. 7A and Supplementary Material, Fig. S10A–C). In PGC-1α KO MEFs, the protein steady-state levels remained unchanged indicating that the drug-mediated activation of mitochondrial biogenesis was blunted (Fig. 7A and Supplementary Material, Fig. S10A–C).

With respect to the starvation program, MEFs exhibited a pattern distinct from HeLa and neuronal cells underlining again the cell-type-specific action of the assessed pharmaceuticals (Fig. 7B and Supplementary Material, Fig. S11A–C, Table 1).

Table 2. Comparison of effects of bezafibrate in vitro and in vivo

<table>
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<tr>
<th>Effect/drug</th>
<th>Sample</th>
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<td>Lysine acetylation</td>
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<td>Starvation signaling</td>
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<td>pAMPK</td>
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<td>Inflammation signaling</td>
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<td>Stress markers</td>
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<td>↑ (PGC)</td>
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PGC, PGC-1α dependent; n.d., not determined.

*P. 100 mg/kg/days, 7 days.

Figure 6. Bezafibrate injection results in a tissue-specific modulation of stress response and post-translational modification. Quantification of (A) PGC-1α and its targets NRF1 and TFAM as well as (B) cellular signaling pathways (mTOR, AKT, AMPK), inflammation (NFκB, IκB and COX2) and general stress response (H01, Hsp70 and SOD2) in lysates of brain, heart, skeletal muscle and liver of mice injected with bezafibrate or vehicle. *P < 0.05; **P < 0.01; ***P < 0.001.

For both PPAR agonists bezafibrate and rosiglitazone, we observed a decrease in mTOR activation and increase in AMPK activation in WT-MEFs. Metformin treatment did also increase AMPK phosphorylation, while mTOR phosphorylation was not altered in WT MEFs. In PGC-1α KO MEFs, the drug-induced alterations were abolished suggesting a regulatory role of PGC-1α in the observed alteration of the metabolic response in MEFs. Interestingly, we observed that some of the levels of phospho-AMPK were drastically decreased in PGC-1α deficient MEFs, indicating that PGC-1α affects AMPK activation already in the untreated state. A possible regulatory role of PGC-1α in the control of the starvation response upon drug treatment is also evidenced by the finding that mTOR-substrate
phosphorylation was decreased in KOs compared with WT MEFs already in the untreated situation. While 4mTOR-substrate phosphorylation increased with increasing concentration in response to bezafibrate and metformin, a decrease in mTOR-substrate phosphorylation was observed in PGC-1α KO cells which is in line with the results on mTOR activation. Rosiglitazone treatment did not induce a change in mTOR-substrate phosphorylation and this effect was not changed by loss of PGC-1α (Supplementary Material, Fig. S12). However, Rosiglitazone treatment of PGC-1α KO MEFs resulted in a decreased pAKT/AKT ratio that remained unaltered in response to the drug in WT MEFs. Increasing concentration of metformin resulted in an increase in pAKT/AKT in WT MEFS, which was blunted in PGC-1α deficient MEFs. In bezafibrate-treated cells, absence of PGC-1α had no effect on AKT phosphorylation (Supplementary Material, Fig. S11A–C).

Drug treatment in WT MEFS also resulted in a robust increase in protein levels of markers of the inflammatory response (Fig. 7B and Supplementary Material, Fig. S11A–C). However, in all analyzed conditions, PGC-1α deficiency abolished the drug-induced alterations, implying a modulatory role of PGC-1α in this program. Similarly, HSP70 protein levels were decreased in PGC-1α KO MEFs when compared with untreated WT MEFs. Moreover, PGC-1α KO lead to a higher level of lysine acetylation in all assessed conditions MEFs. This increase in global protein acetylation was not altered by the drug treatment (Supplementary Material, Fig. S12). While the molecular basis of this finding is yet unclear, this result hints to a regulatory function of PGC-1α in the general cellular stress response.

DISCUSSION

Previous studies, including our own work, have demonstrated a therapeutic benefit of pharmaceutical PGC-1α activation in disorders associated with mitochondrial dysfunction in cellular and murine models (9,11,12,15,19,22,23). In some cases, this benefit seems to be clearly associated with boosting of mitochondrial function. However, in some cases, the molecular basis of the treatment’s success is not clear and tissue- and cell-type-specific effects might play an important role. Considering that mitochondrial dysfunction can cause other cell-wide disturbances and that
High drug concentrations decreased the analyzed markers or showed signs of toxicity (see resveratrol) in HeLa and in neuronal cells. Resveratrol tolerance in neuronal cells was also lower than in HeLa cells. These findings indicate that each cell type might have an individual optimal working range and that high concentrations cause toxicity and therefore limit the potential ‘therapeutic window’. For proliferating healthy cells, decreases in cell survival rate and ATP production in response of high resveratrol and AICAR concentrations have been reported, indicating toxicity when cells are either not undergoing degenerative processes or are challenged (40,41).

A remarkable difference in cell-type-specific effects was observed in the analysis of the starvation program using phosphorylation of mTOR, AMPK and AKT as readout. In this case, the responses of neuronal cell lines, MEFs and HeLa cells to the drug treatment differed (Table 2), indicating that the respective pathways might be regulated on a cell-type-specific level, leading to different and sometimes opposing drug treatment responses. Drug-specific effects might be a result of different target pathways but might also be a result of off-target effects or toxicity (as observed at higher drug concentrations). Similarly, ambiguous effects were sometimes observed when inflammatory and stress markers were assessed. While some drugs activated these responses (such as bezafibrate in neuronal cells) (23), other drugs had no effect or even diminished the response. In MEFs, PGC-1α apparently plays a central role in the regulation of the cellular stress response, as PGC-1α deficiency not only drastically changed marker protein levels (e.g. COX2, Hsp70 or phosphorylated AMPK), but also blunted the drug-induced alteration implying that PGC-1α is the mediator of the drug-stimulated modulation. The cell-type- and drug-specific effects of this scenario make an understanding of the action spectrum and the therapeutic potential of a certain drug very complex and might be linked to tissues-specific effects of PGC-1α.

The strongest differences were observed when the effects of bezafibrate treatment in mouse were compared with our findings in different cell systems (Table 2). The effects of bezafibrate on mitochondrial biogenesis in different tissue and mouse models are under dispute (9,12,15,19,22,23). While the therapeutic effect seems to be clear in several disease models, the exact effect of bezafibrate and the molecular basis is far from understood. The effects of bezafibrate on many other cellular and mitochondrial processes in the disease state, including inflammatory signaling, have been described previously (9,12,15,19,22,23). With this study, we have started a systemic approach to describe the action spectrum of bezafibrate in different tissues. This contributes to the discussion of bezafibrate effects in the complete organism. To dissect effects initiated by a degenerative condition, we injected bezafibrate in a 7-day routine in wild-type mice to understand the effect of the drug itself under basal conditions. We observed a strong tissue-specific effect, which might be contributed to a potential change in concentration of bezafibrate in the different organs. While the PGC-1α pathway and its targets were activated in muscle and liver, no activation was observed for the analyzed regimen in the brain as reported before. In the heart, PGC-1α levels even decreased in response to bezafibrate administration. Mitochondrial markers were selectively increased in liver without any significant effect in other tissues. As we have previously reported in a comparison of bezafibrate injection and oral administration in brain tissue.
[23], it seems plausible that different treatment regimens could lead to different outcomes. This might be especially true for skeletal muscle, where we observed PGC-1α pathway activation after bezafibrate injection without affecting mitochondrial marker proteins. A longer treatment time might be necessary to resolve these parameters in the context of a full organism.

Bezafibrate had the most remarkable effect on markers of the cellular stress response in a tissue-specific manner; similar to observations in the cellular system. Importantly, we observed effects in all analyzed tissues, suggesting that bezafibrate can directly or indirectly (e.g. through behavioral or systemic changes) modulate the function of these tissues. Inflammatory signaling pathways were specifically affected in brain and muscle tissue which is in line with our previous reports of the anti-inflammatory effect of bezafibrate and PGC-1α activation [23]. Starvation signaling including AMPK and AKT was only affected in liver and heart tissue, demonstrating the cell type specificity as observed in our cellular models. Hsp70 was the most sensitive marker for bezafibrate injection in mouse and was increased in brain, liver and skeletal muscle of injected animals indicating an induction of a cellular stress program caused by the drug. Interestingly, this marker was unaffected in our cellular models. Another striking difference between our cellular and murine models was found analyzing lysine acetylation: while no effect of bezafibrate was observed in cells, but increased lysine acetylation in response to bezafibrate injection was detected in liver, skeletal muscle and heart tissue of injected animals.

In conclusion, our analysis of the cell-type- and tissue-specific effects of potential PGC-1α activating drugs revealed that in addition to mitochondrial processes, cellular stress programs can be modulated as well and that in MEFs, the observed effects were clearly dependent on PGC-1α. These findings underline PGC-1α and its pharmaceutical activation as an important stress regulator and warrant future work on the molecular basis and tissue specificity of PGC-1α’s action spectrum and PGC-1α dependent and independent actions to fully comprehend the strong cell-type-specific alterations observed here. Our work highlights changes after treatment with potential PGC-1α-activating drugs in healthy, unchallenged cells and provides crucial data to understand the preconditioning effect of some of the analyzed drugs. The strong tissue- and cell-type-dependent effects and the comparison between in vitro and in vivo effects demonstrate the challenge in predicting the outcome of drug treatment. The knowledge on the tissue- and cell-type-specific action spectrum of the different drugs is thus essential for designing a treatment strategy in degenerative conditions to formulate a deficit-specific compensation strategy and thus a future personalized therapeutic approach. The description of the heterogeneity of the drugs can thus be relevant for designing pre-clinical studies for pathologies where PGC-1α emerges as a potential drug target such as in neurodegenerative diseases and mitochondrial disorders. A comprehensive knowledge on the dose and cell type dependence of the drug can facilitate the evaluation of an individualized drug treatment strategy.

MATERIAL AND METHODS

Cell culture work

SH-SY5Y, HeLa and MEF cells were cultured at 37°C in humidified 5% CO2 and 95% air. HeLa cells and MEFs were grown in DMEM medium supplemented with 10% fetal bovine serum, 1 mM pyruvate and 50 μg/ml uridine. HeLa cells were treated for 72 h either with bezafibrate (Sigma-Aldrich, B7273; dissolved in DMSO), rosiglitazone (ICS International Clinical Service GmbH, S2505; dissolved in DMSO), AICAR (Toronto Research Chemicals, A611700; dissolved in water), metformin (Sigma-Aldrich, D150959; dissolved in water) or resveratrol (Sigma-Aldrich, R5010; dissolved in ethanol) or with the respective vehicle only. SH-SY5Y cells were differentiated (medium containing 1% FBS and 1% P/S and 10 μM all trans-retinoic acid) for 7 days according to a standard protocol [42,43] and treated either with the different drugs or with the respective vehicle for a total of 5 days (see figure legends and text for details on the concentrations).

Animal husbandry

Wild-type C57bl/6 mice (6 weeks old, males) were held in a 12/12 h light/dark cycle at room temperature. For injection experiments, bezafibrate (100 mg/kg/day) was injected i.p. daily for 7 days, and tissue was extracted after PBS perfusion. This study was carried out in strict accordance with the recommendation of German Animal Protection laws. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Office for Nature, Environment and Protection, North Rhine-Westphalia (Landesamt für Natur, Umwelt und Verbraucherschutz). All efforts were made to minimize suffering.

Western blots

Western blot analysis was performed as described on cell and tissue lysates [19,20,23]. Detailed antibody information can be found in Supplemental material. Phosphorylation and lysine acetylation and tissue protein levels were assessed by western blotting of cell lysates following densiometric analysis using ImageJ [44].

BN-PAGE and metabolic chase

BN-PAGE was carried out as described elsewhere to resolve fully assembled OXPHOS complexes in a lauryl maltoside extract as well as supramolecular assemblies of OXPHOS complexes in a digitonin extract [45,46]. Metabolic chase of OXPHOS complexes and supercomplexes was carried out as described following extraction of OXPHOS proteins [47].

Immunostaining and quantification of changes in mitochondrial morphology

Cells were grown on cover slips, fixed in 4% PFA for 10 min at RT, permeabilized with 0.3% Triton X-100 for 5 min at RT and blocked with 3% BSA for 1 h at RT. Cells were labeled with either anti-TOM20 (dilution 1:500) or anti-cytochrome c (dilution 1:800) and anti-tubulin (dilution 1:500) for 2 h at RT following decoration with ALEXA-Fluor 488 or 555 labeled secondary antibodies (dilution 1:500) for 1 h at RT. Cover slips were mounted with ProLong Gold Antifade reagent with Dapi (Invitrogen) and the fluorescence was analyzed with a Delta Vision Olympus IX71 microscope. Images were processed with SoftWorx.
Changes in mitochondrial morphology were quantified by analyzing the area distributions of identified mitochondria components. First, individual cells were selected from our microscopy datasets using ImageJ (44). Image data of selected cells were used to identify mitochondrial components. First, individual cells were selected from our microscopy datasets using ImageJ (44). Image data of selected cells were then loaded into Matlab (R2011b, Image Processing & Statistics Toolbox, Mathworks, Inc.). Prior to the identification (segmentation) of mitochondria, images were normalized and a sequence of filters was applied (unsharp, tophat, median) to improve segmentation results. Filter settings were manually chosen and remained constant on all processed images (unsharp: 3 × 3 kernel; tophat: disk-shaped structuring element with radius of 7 pixel; median filter: 3 × 3 kernel). After preprocessing, mitochondria were segmented using Otsu’s thresholding method. Segmentation results were then manually controlled for consistency and quality. For simple quantification, we chose to categorize identified mitochondria components into three classes (small, medium, large). Thresholds for individual classes were obtained by estimation of an expert observer. Two metrics were obtained for each class/experiment: (1) percent components: number of components in class divided by total number of components (per cell) and (2) percent mitochondria area: sum of component area in class divided by total mitochondria area in the cell. For comparison between control and treatment experiments, a two-tailed t-test was performed for each class and metric.

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG online.

Conflict of Interest statement. None declared.

FUNDING
This work was supported by the Emmy-Noether-Program of the Deutsche Forschungsgemeinschaft (WE 4108/3-1 to T.W.), the Cluster of Excellence: Cellular Stress Responses in Aging-Associated Diseases (CECAD to A.S., N.K., A.H. and T.W.), Collaborative Research Center SFB 635 (to T.W.), the Fritz-Thyssen-Foudnationa (to T.W.), the Care-for-Rare-Foundation (to T.W.) and by the Bundesministerium für Bildung und Forschung (BMBF, mitoNET-Deutsches Netzwerk für mitochondriale Erkrankungen, 01GM1113B to T.W.).

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