Aging and anti-aging: Unexpected side effects of everyday medication through sirtuin1 modulation

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Abstract. The sirtuin 1 protein (SIRT1) is a member of the class III NAD$^+$-dependent histone deacetylases, which are also referred to as the 'sirtuins'. The sirtuins and silent information regulator 1 (SIRT1) in particular, are known to play a role in the response to DNA damage, metabolism, longevity and carcinogenesis. SIRT1 regulates different cellular processes such as proliferation, differentiation and apoptosis through deacetylation of important regulatory proteins such as p53, FOXO3a and NFκB. A number of different modifiers of SIRT1 expression and activity have been discovered and even food and cosmetic additives (e.g. resveratrol and dihydrocoumarin) have been suggested to either activate or inhibit the activity of human SIRT1. We screened a panel of 18 different drugs which are frequently used in everyday clinical practice with regard to their influence on cell survival and SIRT1 expression in freshly isolated peripheral blood mononuclear cells (PBMCs) from young and healthy volunteers. In this context, we identified L-thyroxin, insulin and sodium nitroprusside to be potent activators of human SIRT1 expression. In addition, treatment of PBMCs with sodium nitroprusside was associated with a significant cellular lifespan extension, while L-thyroxin and insulin were unable to prolong lifespan, suggesting that isolated upregulation of SIRT1 is in fact insufficient to promote longevity. These findings have an important impact on the long-term use of a number of frequently used clinical agents in the treatment of chronic disease with respect to aging and carcinogenesis.

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Abbreviations: SIRT1, silent information regulator 1; HDAC, histone deacetylase; HAT, histone acetyltransferase; NAD, nicotinamide adenine dinucleotide

Key words: silent information regulator 1, histones, acetylation, aging, L-thyroxin, insulin, nitric oxide

Introduction

Reversible DNA and protein modification, such as acetylation and deacetylation, are involved in the regulation of transcriptional activity and influence protein enzymatic activity. Acetylation of histone N-terminal lysine residues is carried out by histone acetyltransferases (HATs) and is associated with increased transcriptional activity in eukaryotic cells through chromatin relaxation and may be reversed by competing histone deacetylase (HDAC) enzymatic activities, which cause histone deacetylation and thus chromatin condensation and subsequently repression of gene expression (1,2). An imbalance of this highly sensitive equilibrium between acetylation and deacetylation is associated with malignant diseases such as prostate cancer (3), acute myeloid leukemia (4) and other solid tumors and hematological malignancies. Based on structural and functional similarities to their ancestor proteins in yeast RPD3, HDA1, and SIR2, HDACs are divided into four distinct classes. Class I HDACs include the RPD3 homologs HDAC1, 2, 3, and 8. Class II HDACs contain the HDA1 homologs HDAC4, 5, 6, 7, 9, and 10, while the class III HDACs (also referred to as the sirtuins) share a significant degree of homology with the yeast histone deacetylase SIR2, comprising the human proteins SIRT1-7 (5,6). The class IV HDACs includes HDAC11, the only protein known of this class.

Human SIRT1, which is a member of the conserved SIR2 family of NAD$^+$-dependent histone deacetylases, is localized in the nucleus and is known to deacetylate both histone proteins (with preference for histone H4 lysine 16) and non-histone proteins (p53, FOXO3a, NFκB, PCG1α) (Fig. 1) (7-12). Histone H3 acetylated lysine residues in position 9 and 14 and, to a greater extent, histone H4 lysine 16 are preferential SIRT1 histone targets (8,13). Every single deacetylation by SIRT1 is connected with the hydrolysis of one NAD$^+$ to ADP-ribose and nicotinamide for each acetyl group that is being removed (14).

SIRT1 is known to be implicated in various cellular processes such as metabolism, differentiation, response to DNA damage and apoptosis (10,12,15-18). It has been suggested to be linked to caloric restriction, cellular energy status and longevity in numerous species from yeast to rodents (Fig. 1). In yeast, caloric restriction is associated with increased levels of NAD$^+$ and an activation of SIR2, which in

turn increases rDNA stability. In this context, the formation of toxic extrachromosomal rDNA circles is reduced, which is consequently associated with an extension of cellular lifespan (19). SIRT1 mediates antiapoptotic effects through deacetylation of the tumor suppressor protein p53 (Lys 382) and the forkhead transcription factor FOXO3a, which are known to be proapoptotic mediators. Cells are therefore increasingly resistant to DNA damaging agents and oxidative stress (7,9,10). In addition, SIRT1 is known to promote TNF-α-induced apoptosis through deacetylation and thus inactivation of NFκB, which is an antiapoptotic transcription factor (Fig. 1) (10).

The human class III HDAC, SIRT1, has been suggested to play a role in the pathogenesis of cellular malignancy since it regulates gene silencing and/or lifespan in multiple organisms, thus sustaining the continued survival of cancerous growth (12,20-22). It also regulates p53 function (9,11,23), and plays an important role in stress signaling (24,25). The human histone deacetylase SIRT1 is a key element in the regulation of longevity and in the pathogenesis of malignant disease. siRNA-mediated SIRT1 knockdown has been reported to induce growth arrest and apoptosis in human epithelial cancer cells, while normal epithelial cells remain unaffected (10,18). In addition, deacetylation of specific SIRT1 targets such as the histone H4 lysine 16 residue has been found in various tumors and dysplastic tissues with increasing loss of tumor differentiation (17). However, to date, SIRT1 has been associated only with the transcriptional repression of mammalian target genes that are already basally expressed (26). In addition, SIRT1 has not been linked to either heritable silencing of tumor suppressor genes or to heterochromatin maintenance (27). The sirtuins have distinct specific inhibitors (28-30) and are not responsive to drugs such as trichostatin-A (TSA) or other class I and II HDAC inhibitors that have been previously used to study promoter-hypermethylated tumor suppressor genes. We, therefore, focused our research efforts on the identification of agents exhibiting the capacity to modulate SIRT1 expression and activity, i.e. inhibitors of SIRT1 such as potential new agents in the treatment of cancer and activators of SIRT1 activity in order to extend lifespan. Nicotinamide (vitamin B3) is a physiological noncompetitive inhibitor of SIRT1. The balance between nicotinamide and NAD^+ is regulated by a multi-functional domain of SIRT1 that is in fact also competent to cleave NAD^+ (28). In yeast, nicotinamide is known to increase rDNA recombination and to shorten replicative lifespan (28,30). To date, a number of SIRT1 inhibitors have been identified; e.g. indols (31), sirtinol (29,32), splitomycin (29,33) and cambinol (18) with potent inhibiting capacity. Notably, also food and cosmetic additives have been shown to modulate SIRT1 activity. Dihydrocoumarin for instance, induces hyperacetylation of p53 through repression of SIRT1 activity thus increasing apoptosis (20). The most celebrated activator of SIRT1, however, is resveratrol, a plant phenol and red wine component, which is already known to be cardio-protective and to work against age-associated neuro-degeneration and tumorigenesis (34-37). In yeast, resveratrol is known to extend lifespan by repressing rDNA recombination and formation of toxic extrachromosomal rDNA circles. Resveratrol, therefore. mimics events that are observed during caloric restriction by activating SIR2. Other potent activators of SIR2 are plant phenols such as piceatannol and quercetin (34), which, however, do not have a significant effect on lifespan.

The fact that a number of food and cosmetic additives have been reported to have an effect on the expression and activity of SIRT1 and thus on lifespan and potentially the pathogenesis of malignant disease, led us to speculate that a number of 'harmless' drugs that are used in everyday clinical practice may also have an influence on human SIRT1 and thus cellular function and survival. In the present study, freshly isolated peripheral blood mononuclear cells (PBMCs) from healthy adult volunteers were used to screen the effect of different drugs on cell survival and SIRT1 expression and activity. We identified insulin, L-thyroxin and sodium nitroprusside to be activators of SIRT1 expression, and we investigated their influence on cell survival and histone H4 lysine 16 acetylation ($H4_{K16}$) status as a specific SIRT1 target.

Materials and methods

Pharmaceuticals. The following pharmaceuticals were used: acetylsalicylic acid (Aspisol® 100 mg/ml, Bayer Vital GmbH/Pharma, Germany), amiodarone (Cordarex® 50 mg/ml, Sanofi Synthelabo GmbH, Germany), clemastine (Tavegil® 400 μg/ml, Novartis Consumer Health GmbH, Germany), dexamethasone (Fortecortin® 4 mg/ml, Merck Pharma GmbH, Germany), diazepam (Diazepam-ratiopharm® 5 mg/ml, Ratiopharm GmbH, Germany), digitoxin (Digimerck® 250 μg/ml, Merck Pharma GmbH), dimenhydrinate (Vomex® 6.2 mg/ml, Astellas Pharma GmbH, Germany), esmolol (Brevibloc® 10 mg/ml, Baxter, Germany), furosemide (Lasix® 10 mg/ml, Aventis Pharma Deutschland GmbH, Germany), haloperidol (Haldol® 5 mg/ml, Janssen-Cilag GmbH, Germany), human insulin (Actrapid® 40 IE, Novo Nordisk Pharma GmbH), L-thyroxin (L-Thyroxin® 100 g/ml, Henning Berlin Arzneimittel, Germany), metamizole (Novalgin® 500 mg/ml, Aventis Pharma Deutschland GmbH), sodium nitroprusside (Nipruss® 6 mg/ml, Schwarz Pharma Deutschland GmbH, Germany), omeprazole (Pantozol® 4 mg/ml, Altana Pharma Deutschland GmbH, Germany), thiamazole (Favistan® 40 mg/ml, Temmler Pharma GmbH & Co KG, Germany), valproic acid (Orfiril® 100 mg/ml, Desitin Arzneimittel GmbH, Germany), thiamine (vitamin B1 50 mg/ml, Ratiopharm GmbH), and heparin (Heparin-Natrium® 25.000 IE, Ratiopharm GmbH).

Isolation of PBMCs. Heparinised venous blood samples were collected from young and healthy adult volunteers aged 18-30 years. PBMCs were isolated immediately by Ficoll density gradient centrifugation (Biochrom, Berlin, Germany) and then washed twice in phosphate-buffered saline (PBS) (Cambrex, Verviers, Belgium). Cells were cultured in RPMI-1640 (Cambrex) containing 10% fetal bovine serum (FBS) (Biochrom) at a density of 10^6/ml.

Cell culture and treatment of PBMCs. After isolation, cells were treated with different agents as mentioned above. The concentration of the drugs was calculated based on the

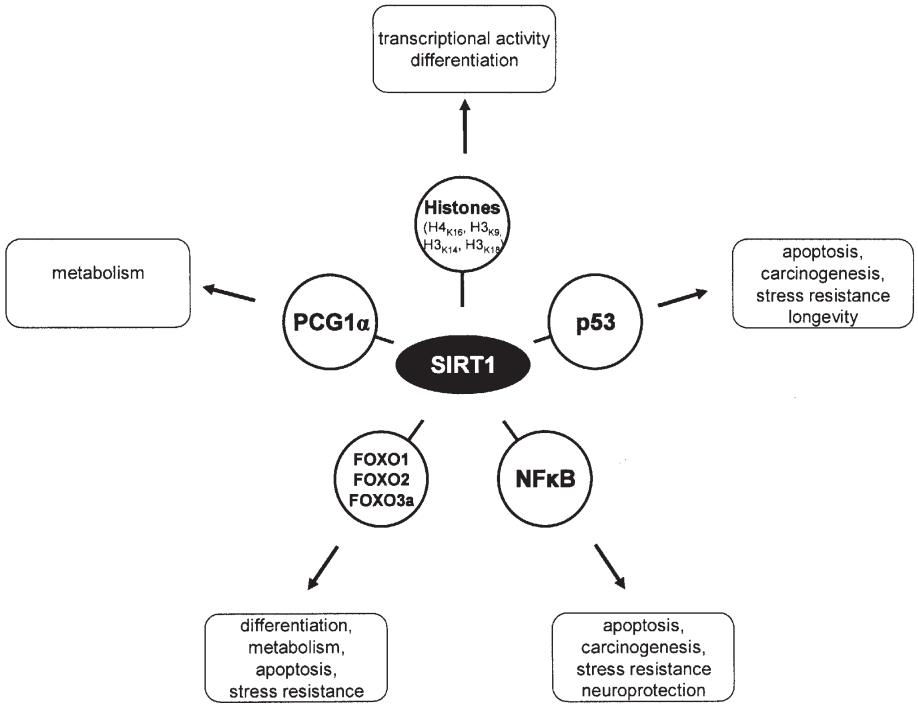

Figure 1. Overview of important interacting histone and non-histone proteins of the sirtuin deacetylase SIRT1. Deacetylation of these SIRT1 targets plays a key role in different cellular processes. Depending on the affected protein, it influences transcriptional activity, apoptosis, longevity, cellular differentiation, metabolism, carcinogenesis and regulation of stress resistance.

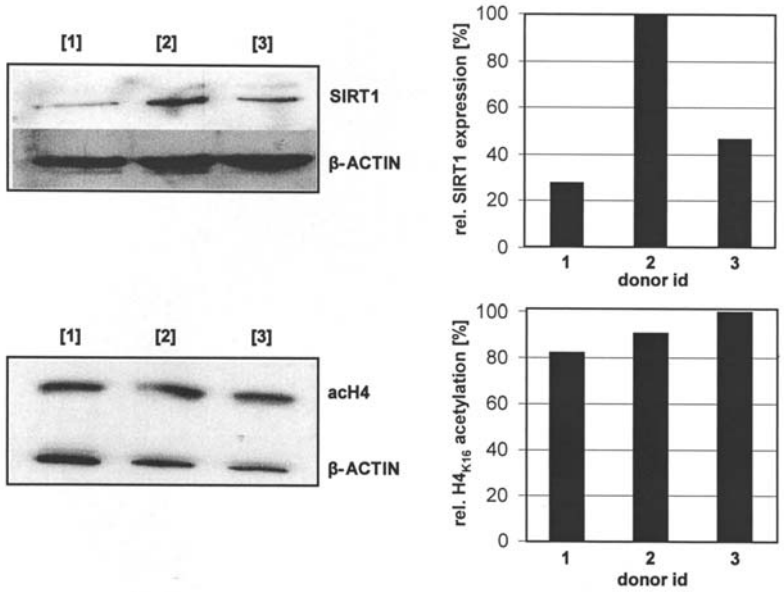

Figure 2. Basal expression of human histone deacetylase SIRT1 in peripheral blood mononuclear cells of 3 different female blood donors (age, 22-27 years) and the corresponding human acetylated histone H4 (lysine 16) ($H4_{K16}$) acetylation status. A comparatively high variability in the basal SIRT1 expression between the 3 donors was not reflected by corresponding changes in the $H4_{K16}$ acetylation status.

intravenous dose that is usually administered to a 70-kg individual assuming that the active component would dispense in a total volume of 5 liters of blood. PBMCs were cultured in RPMI-1640 for a minimum of 8 h and a maximum of 14 days.

Flow cytometry. Flow cytometric analyses were carried out on a Becton Dickinson FACScan® flow cytometer (BD, Heidelberg, Germany). FACS analyses were carried out immediately after cell isolation and after 1, 2, 4, 6, 8, 10, 12 and 14 days of treatment. PBMCs were re-suspended and washed in phosphate-buffered saline (PBS) supplemented with 10% FBS. Cells ($0.5x10^6$) were diluted in 200 μl of PBS (+10% FBS) and stained with 5 μl of propidium iodine (50 μg/ml) (Sigma, Munich, Germany) for 5 min. FACS data were analysed with the WinMDI 2.7 software.

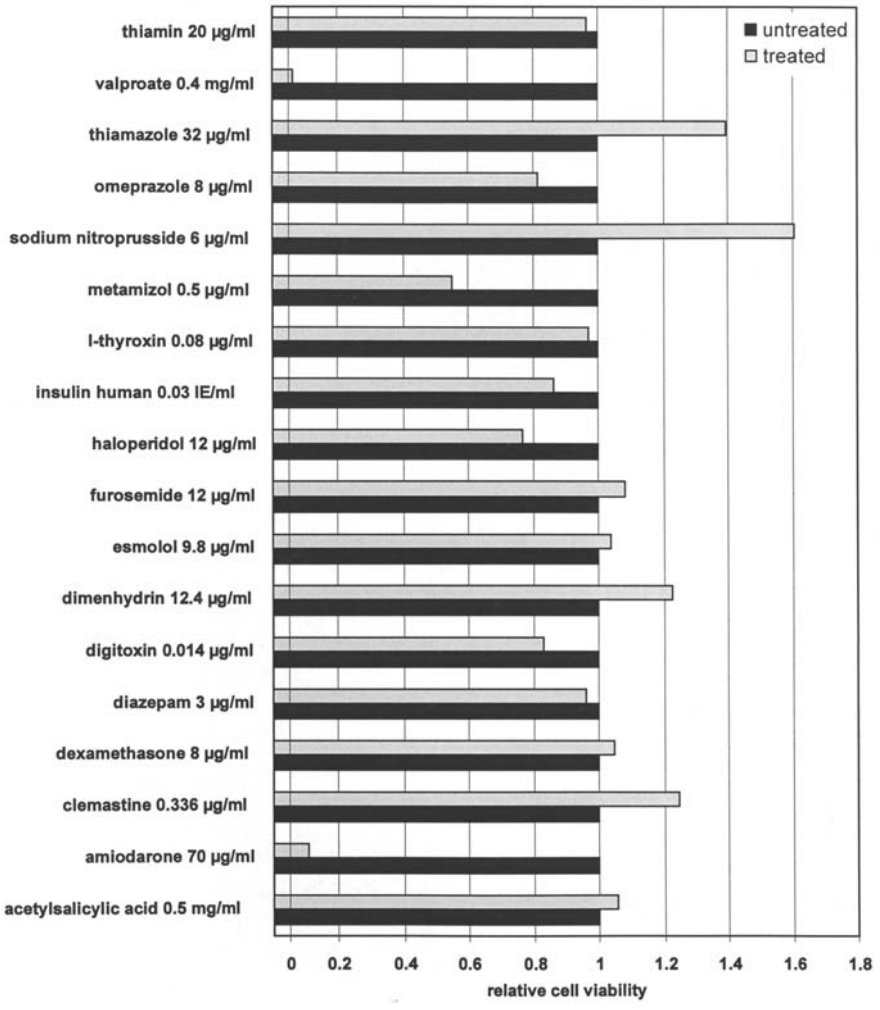

Figure 3. Viability of the untreated cell fraction (=1) compared to the treated cells of one blood donor. After 14 days of incubation with diverse drugs, treatment with sodium nitroprusside showed the greatest effect and revealed an extension of cellular lifespan by ~60% compared to untreated PBMCs.

Protein isolation and Western blot analysis. Eight, 24, 48 (and 72) h after treatment $1x10^6$ PBMCs were lysed using 80 μl of no-salt-lysis-buffer (50 mM HEPES, pH 7.1, % NP40, and 100 μg/ml PMSF). In order to increase lysis efficiency, lysates were frozen at -80˚C for 1 h before centrifugation. Protein extracts were then separated by electrophoresis on 8 or 15% polyacrylamide gels and then transferred to nitrocellulose membranes (BioRad, Munich, Germany). The membranes were then incubated with mouse monoclonal antibodies against the human SIRT1 protein (Upstate Biotechnology via Biomol, Hamburg, Germany) followed by incubation with a peroxidase-conjugated secondary antibody (Dianova, Hamburg, Germany). The immunoreactive proteins were visualized by chemiluminescence.

Histone isolation (acid extraction) and analysis. In parallel to protein isolation, histones were isolated from PBMCs after 8, 24, 48 (and 72) h of treatment. Cells ($2x10^6$) were lysed in 200 μl of lysis buffer (10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, and 0.5 mM DTT). In order to increase lysis efficiency lysates were frozen at -80˚C for 1 h before adding 50 μl of 2 M sulfuric acid dropwise under permanent vortexing. Samples were then left on ice for at least 1 h with intermittent vortexing every 10 min, and then centrifuged for 10 min at 13,000 rpm. Subsequently, proteins were precipitated in three additional volumes of 20% trichloroacetic acid, leaving the samples on ice for 1 additional h and intermittent vigorous vortexing every 10 min. Samples were pelleted by centrifugation for 10 min at 13,000 rpm. Later, pellets were washed twice with acetic aceton (0.1% hydrochloric acid), twice with aceton and finally re-suspended in 25 μl water. Histone samples were analysed by Western blot analysis as described above using rabbit monoclonal antibody against human acetylated histone H4 (lysine 16) ($H4_{K16}$) (Upstate Biotechnology via Biomol). The purity of histone isolates was checked on Coomassie-stained polyacrylamide gels (Coomassie Brilliant Blue®, AppliChem, Darmstadt, Germany).

Results

Basal expression and activity of SIRT1 in different donors. PBMC protein analysis from young and healthy volunteers revealed a high variability in the basal expression of SIRT1. In order to demonstrate this variability, proteins were isolated from PBMCs from similar female donors 22-27 years of age. The variability in the basal expression of SIRT1 is shown in a Western blot analysis (Fig. 2).

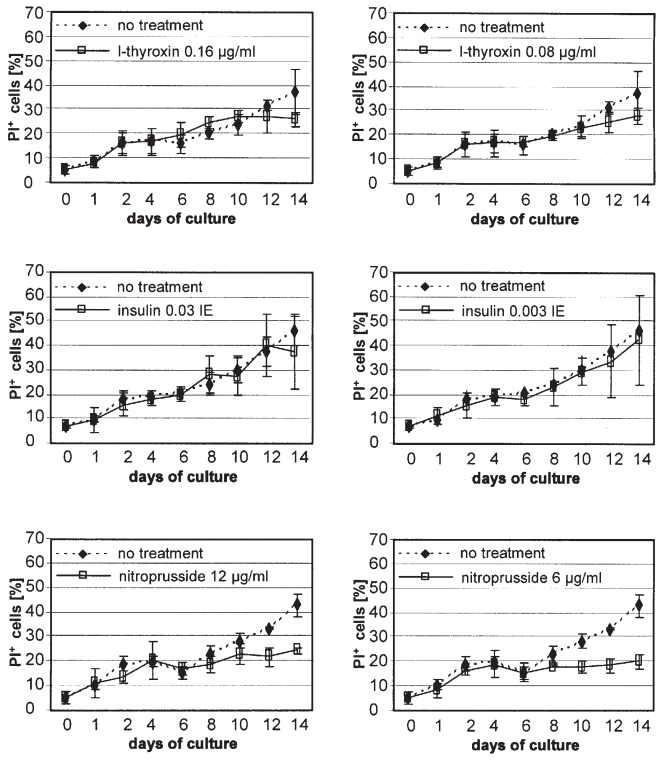

Figure 4. Treatment of PBMCs with two different concentrations of L-thyroxin, insulin or sodium nitroprusside over 14 days compared to untreated cells. FACS analyses were performed directly after isolation and after 1, 2, 4, 6, 8, 10, 12 and 14 days of incubation. Cells were stained with propidium iodide, and cell viability was measured. Each experiment was repeated three times with PBMCs of different donors (average levels and standard deviations are shown). Treatment with L-thyroxin and insulin showed no effect on lifespan extension. However, incubation with sodium nitroprusside prolonged the lifespan of PBMCs by at least 20% absolutely.

In order to determine whether the different protein levels were associated with a variation in the acetylation status of SIRT1, the acetylation status of histone $H4_{K16}$, which is a specific target of SIRT1, was assessed from histones that were isolated from PBMCs in the same experiment. Notably, despite the variability of SIRT1 protein expression, no difference in the $H4_{K16}$ acetylation status was observed (Fig. 2).

Effect of different drugs on cell survival and SIRT1 protein expression. The effect of various drugs on cell survival was assessed in two different ways. First, in order to investigate the functional aspect, treated human PBMCs were cultured for 14 days, and FACS analysis was performed as previously described. In Fig. 3 the results after 14 days of treatment are shown compared to untreated PBMCs. Our analyses on the effect of different agents on cellular lifespan showed that treatment with sodium nitroprusside had a striking effect on cellular lifespan extension after 14 days of treatment, revealing that the NO donor was able to extend cell viability up to 60% when compared to the untreated control. All the other agents showed no or only a poor effect on lifespan extension. Second, to analyze the effect of the agents mentioned above on SIRT1 protein level, cells were treated for 24 h, and Western blot analysis was carried out as described previously. We identified 3 drugs which were able to upregulate SIRT1 protein level after 24 h of incubation: L-thyroxin, insulin and sodium nitroprusside. Following these results, we decided to further analyse the effect of these three pharmaceuticals.

Effect of L-thyroxin on cell survival, SIRT1 protein expression and activity. Peripheral blood mononuclear cells from three different adult volunteers were treated with 0.08 or alternatively 0.16 μg/ml L-thyroxin. After 24 h, 48 h, 4, 6, 8, 10, 12 and 14 days of treatment, cell viability was assessed by flow cytometry and compared with the untreated control. In accordance with the results obtained during the screening analyses, no relevant effect on life extension was observed (Fig. 4). In order to confirm the results that were obtained from the screening studies, protein analyses were repeated and SIRT1 protein levels were assessed after treatment of PBMCs with 0.08 or 0.16 μg/ml L-thyroxin, respectively. Histones were isolated from the same experiment, and the $H4_{K16}$ acetylation status, which is a specific SIRT1 target, was determined (Fig. 5). When PBMCs were treated with L-thyroxin, SIRT1 protein levels were increased by 3- to 5-fold in a dose-dependent manner after 24 h of incubation. After 8 and 48 h of treatment, no such effect was observed. Concurrent with the upregulation of SIRT1 expression after 24 h, the $H4_{K16}$ acetylation level was decreased by up to 50% in the presence of L-thyroxin, while lower concentrations of the agent (0.08 μg/ml L-thyroxin) was associated with a more pronounced deacetylation. Notably, despite the rapid decline of SIRT1 at the protein level after 24 h of incubation, increased deacetylation persisted for more than 24 h. These results indicate that both expression and activity of the human histone deacetylase SIRT1 were upregulated in the presence of L-thyroxin.

Effect of insulin on cell survival, SIRT1 protein expression and activity. As for L-thyroxin, FACS analysis was also performed on cells that were exposed to insulin. After 14 days of treatment, insulin showed no effect on lifespan extension (Fig. 4).

In the next step we analysed SIRT1 protein levels after treatment with insulin for 8, 24, 48 and 72 h, and we found that insulin upregulated SIRT1 protein expression with a peak after 24 h and a slow decline over time up to 72 h (Fig. 6). In parallel to protein isolation, histones were isolated from PBMCs that had been treated with insulin. Insulin treatment, however, did not have an effect on the acetylation status of histone H4 (lysine 16) (Fig. 6) despite the increased expression of SIRT1.

Effect of sodium nitroprusside on cell survival, SIRT1 protein expression and activity. Sodium nitroprusside was found to be the only agent that did in fact extend PBMC lifespan by at least 20%. These results were verified in at least 3 independent experiments in PBMCs from healthy adult volunteers (Fig. 4). SIRT1 protein expression was assessed by Western blot analysis in PBMCs that had been treated with sodium nitroprusside for 8, 24, 48 and 72 h, respectively. We identified a significant variability among individuals with respect to the effect of nitroprusside on SIRT1 protein expression. Fig. 7 shows Western blot analyses from two different blood donors (1 and 2). SIRT1 enzymatic activity subsequent to nitro-

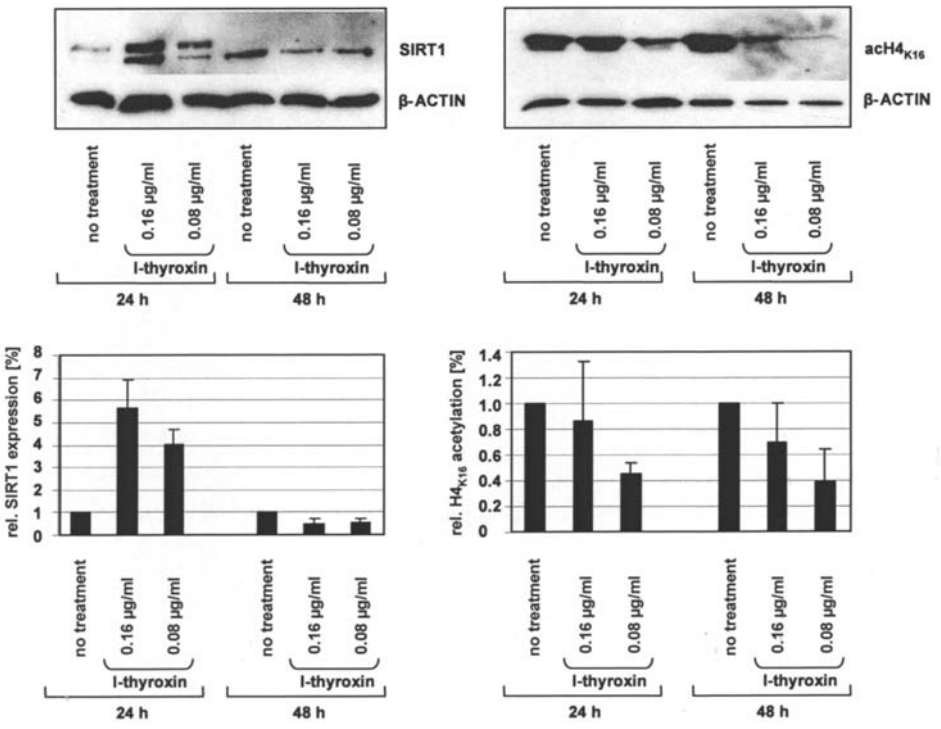

Figure 5. PBMCs from 3 different healthy blood donors were treated with either 0.16 or 0.08 μg/ml L-thyroxin besides the average level (normalised on the level of untreated cells), and one representative Western blot is shown. After 8, 24 and 48 h of incubation, protein and histones were isolated, and the levels of SIRT1 expression and $H4_{K16}$ acetylation were detected. SIRT1 expression was upregulated by thyroxin treatment after 24 h of incubation, however after 8 (data not shown) and 48 h no effect was observed. Furthermore, $H4_{K16}$ deacetylation was observed.

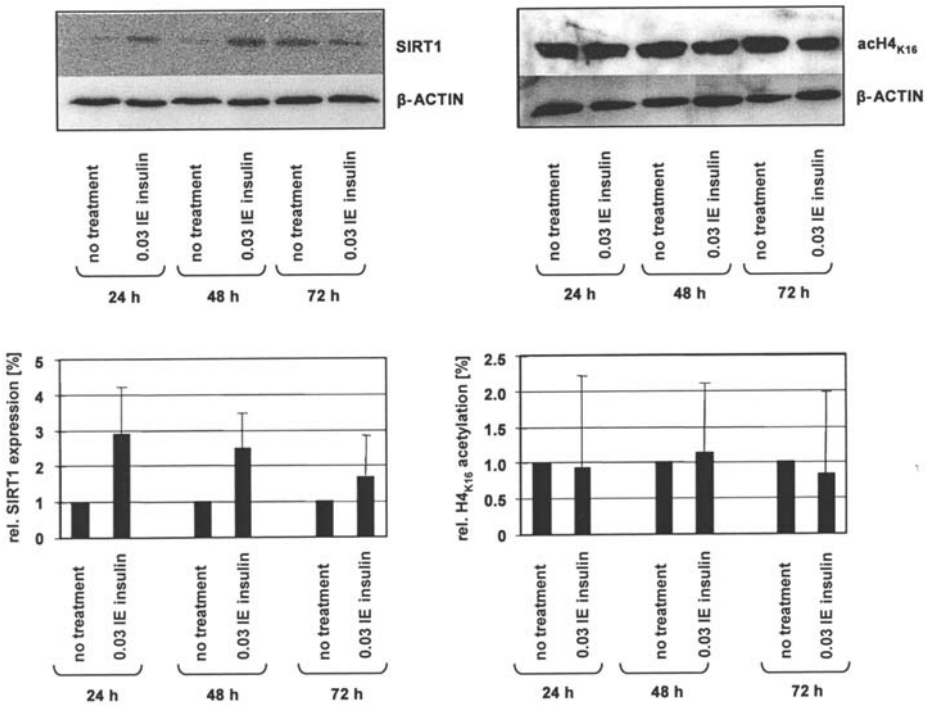

Figure 6. PBMCs from 3 different blood donors were treated with 0.03 IE insulin, besides the average level (normalised on the level of untreated cells), and one representative Western blot is shown. After 8, 24, 48 and 72 h of incubation, protein and histones were isolated, and the levels of SIRT1 expression and $H4_{K16}$ acetylation were measured. SIRT1 expression was upregulated by insulin after 24 h of incubation, however after 8 h no effect was observed (data not shown). Protein levels slowly declined over a period of up to 72 h. An effect of insulin on $H4_{K16}$ acetylation was not observed.

prusside treatment was further determined on histone isolates where the acetylation status of the SIRT1 target $H4_{K16}$ was analysed (Fig. 7); increased SIRT1 protein levels in PBMCs from donor 1 showed a decrease in the acetylation status after 24, 48 and 72 h of treatment. By contrast, PBMCs from donor 2 showed decreased $H4_{K16}$ acetylation levels after 24

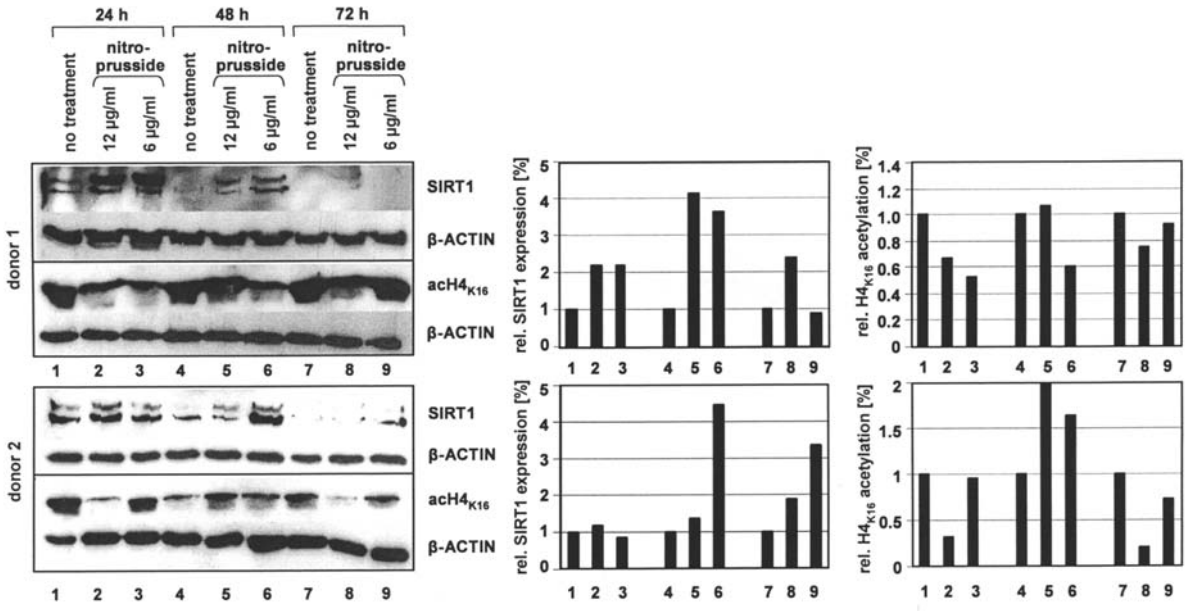

Figure 7. PBMCs from 3 different blood donors were treated with either 12 or 6 μg/ml sodium nitroprusside for 8, 24, 48 and 72 h. The results obtained from 2 donors are shown. After 8 h of incubation no effect was observed (data not shown). Calculating the average level seemed to be implausible because of the significant inter-individual variability. It was observed that nitroprusside induced SIRT1 protein expression and activity. However, the point of maximum induction, the level of induction, and the sensitivity for different concentrations of this agent varied significantly among different individuals.

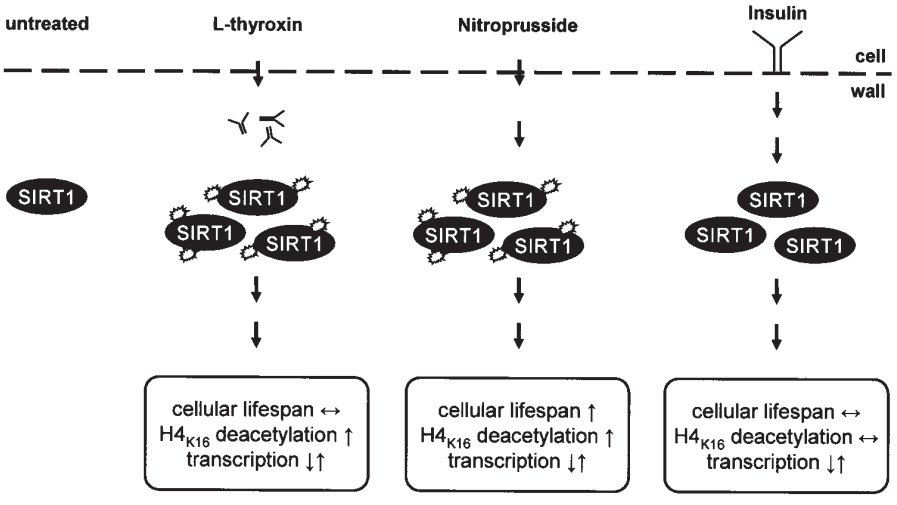

Figure 8. Effect of L-thyroxin, nitroprusside and insulin on SIRT1. L-thyroxin as well as nitroprusside induced SIRT1 expression and SIRT1 activity (measured by increased deacetylation of the SIRT1 target $H4_{K16}$), while incubation with nitroprusside but not with L-thyroxin was able to prolong the lifespan of PBMCs, suggesting that isolated activation of SIRT1 is not sufficient to promote longevity. Insulin treatment also increased SIRT1 expression but did not have an effect on the $H4_{K16}$ acetylation status. In light of their influence on activity and/or expression of SIRT1, L-thyroxin, nitroprusside and insulin may have an impact on transcriptional activity of various important genes.

and 72 h of treatment, even though 48 h was needed to induce SIRT1 expression. In conclusion, SIRT1 expression and activity were strongly induced by sodium nitroprusside.

Discussion

The posttranslational modification of histones and non-proteins through reversible acetylation has been recognized to be a potential molecular target in cancer therapy. Class I and II HDAC inhibitors of different chemical origin are increasingly becoming part of established treatment regimens for solid and hematological tumors (38,39). Significant attention is currently being drawn to the contribution of class III HDACs (sirtuins) in tumorigenesis, neurodegeneration, aging and age-related disease (17,40-42). Research efforts of our own and several other groups have been conducted to identify new modulators of sirtuin activity and thus histone and protein acetylation from a panel of agents in everyday clinical practice in order to provide new indications for these agents in areas such as anti-aging and tumor therapy with a tolerable minimum number of side effects (18,29,32,34). Apart from SIRT1 inhibitors such as nicotinamide and

cambinol, food and cosmetic additives have also been shown to operate as activators (e.g. resveratrol) or inhibitors (e.g. dihydrocoumarin) of SIRT1 (20,34).

In the study presented herein, we investigated the effect of various clinical agents on cell survival and the expression and activity of the NAD^+-dependent histone deacetylase SIRT1. After screening 18 different drugs which are frequently used and which represent an integral part of everyday clinical therapy, we identified L-thyroxin, insulin and sodium nitroprusside to be activators of SIRT1 expression (Fig. 8). Among these, insulin increased SIRT1 expression without any influence on the $H4_{K16}$ acetylation status, which represents a specific SIRT1 target without affecting cell viability. The absence of $H4_{K16}$ deacetylation may be caused by various reasons such as a difference in SIRT1 substrate specificity in the context of different cellular circumstances. L-thyroxin functions as a dose-dependent activator on the expression and the activity of SIRT1, while cell survival is unaffected. Depending on the blood donor, sodium nitroprusside was able to upregulate SIRT1 expression and activity. In addition, nitroprusside, which is an NO donor, was able to extend lifespan after 14 days of treatment by at least 20% when compared to the untreated control.

The insulin signaling pathway and SIRT1 are known to be involved in the regulation of aging. Mutations within the insulin/insulin-like-growth-factor (IGF) signaling pathway have been suggested to be accompanied by lifespan extension, increased peripheral insulin sensitivity and stress resistance in yeast, worms, fruit flies and rodents (43-45). Alterations of the insulin/insulin-like-growth-factor signaling pathway have been linked to human aging as well. While decreased IGF levels and genetic aberrations within the IGF-receptor, due to the minimization of general mitogenic stimuli, are associated with reduction of age-related pathologies and old age (46,47), excessive IGF levels have been reported to be associated with abnormal proliferation, metabolic dysfunction and increased incidence of malignant disease (48,49). Our observations of insulin being a potent activator of SIRT1 expression and thus a mediator of proliferation and metabolic dysfunction concur with the findings mentioned above.

L-thyroxin was the second hormone that we identified to have an influence on SIRT1 expression. Unlike insulin, L-thyroxin diffuses through the cell membrane in order to bind to its intranuclear receptor. Intranuclear receptors such as the thyroxin-binding protein play an important role in the physiological regulation of growth and differentiation (50,51). Our results strongly suggest a direct link between SIRT1 and L-thyroxin; SIRT1 expression was induced by L-thyroxin and was accompanied by deacetylation of $H4_{K16}$. Notably, SIRT1, itself, which is associated with lifespan extension and longevity in organisms ranging from yeast to rodents (19,41,52) is decreased as aging progresses (8). Thyroid hormones are therefore gaining increasing attention as potential markers of aging (53). It is known that aging is accompanied by altered thyroid function and decreased concentrations of peripheral T3, T4 and TSH levels, while thyroxin signaling is reduced as a consequence of decreased concentrations of intranuclear thyroxin receptors at the same time (54,55). Our results, therefore, imply that thyroxin is part of the physiological aging process that is mediated by SIRT1; reduced concentrations of the thyroxin-binding protein and thus the absence of thyroxin-mediated signaling directly cause a decrease in SIRT1 expression and the propagation of the aging process.

As mentioned above, the NAD^+-dependent histone deacetylase SIRT1 is known to extend lifespan (19,41,52). It is, however, noteworthy, that out of the three agents that were identified to induce SIRT1 expression, only sodium nitroprusside was in fact able to prolong the lifespan of PBMCs. These data are consistent with observations published by Michishita *et al* (8) concerning stably overexpressed SIRT1 and other sirtuins in normal human fibroblasts and epithelial cells, which were not associated with any signs of lifespan extension in normal human cells either. Thus the isolated overexpression of SIRT1 may not be sufficient to mediate longevity in normal human cells.

Lifespan extension after treatment with sodium nitroprusside was previously observed by other authors, and mediating mechanisms may at least, in part, be explained by s-nitrosylation and thus caspase inactivation (particularly caspases 1 and 3) (56,57). Antioxidative effects such as neutralization of reactive oxygen species of the NO donor seem implausible after treatment for 14 days and in light of the application of only a small dose of sodium nitroprusside at the beginning of the experiment. Additionally, sodium nitroprusside has a half-life in biological systems ranging between 2 and 3 sec only (58). In support of our findings of NO acting as an inductor of SIRT1, Nisoli and coworkers (59) reported the induction of endothelial NO synthase and increase of SIRT1 expression in various tissues in mice following caloric restriction. Our results confirm and further support previous conclusions suggesting that NO is also a SIRT1-inducing component in human PBMCs thus altering the intracellular acetylation status and consequently transcriptional activity. We conclude that the treatment of human PBMCs with agents frequently used in everyday clinical practice such as L-thyroxin, insulin and sodium nitroprusside, have the capacity to alter expression and activity of the human NAD^+-dependent histone deacetylase SIRT1, leading to an alteration of the intracellular acetylation status and thus the transcriptional activity of numerous genes that are involved in fundamental regulation processes such as proliferation, differentiation and apoptosis. These modifications may have important consequences in the long-term treatment of patients and may lead towards further elucidation of the pathogenesis of atherosclerosis, carcinogenesis and age-related disease.

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