The Creatine Kinase System in Human Skin: Protective Effects of Creatine Against Oxidative and UV Damage *In Vitro* and *In Vivo*

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Cutaneous aging is characterized by a decline in cellular energy metabolism, which is mainly caused by detrimental changes in mitochondrial function. The processes involved seem to be predominantly mediated by free radicals known to be generated by exogenous noxes, e.g., solar ultraviolet (UV) radiation. Basically, skin cells try to compensate any loss of mitochondrial energetic capacity by extra-mitochondrial pathways such as glycolysis or the creatine kinase (CK) system. Recent studies reported the presence of cytosolic and mitochondrial isoenzymes of CK, as well as a creatine transporter in human skin. In this study, we analyzed the cutaneous CK system, focusing on those cellular stressors known to play an important role in the process of skin aging. According to our results, a stress-induced decline in mitochondrial energy supply in human epidermal cells correlated with a decrease in mitochondrial CK activity. In addition, we investigated the effects of creatine supplementation on human epidermal cells as a potential mechanism to reinforce the endogenous energy supply in skin. Exogenous creatine was taken up by keratinocytes and increased CK activity, mitochondrial function and protected against free oxygen radical stress. Finally, our new data clearly indicate that human skin cells that are energetically recharged with the naturally occurring energy precursor, creatine, are markedly protected against a variety of cellular stress conditions, like oxidative and UV damage *in vitro* and *in vivo*. This may have further implications in modulating processes, which are involved in premature skin aging and skin damage.

Key words: cellular energetics/creatine/creatine kinase/mitochondria/skin aging/UVA irradiation
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According to a widely accepted theory of aging, alterations in mitochondrial function can be regarded both as an important cause, as well as a consequence of aging (Harman, 1956). A lack of mitochondrial function impairs cellular ATP synthesis, and altered mitochondrial physiology can additionally induce the formation of free reactive oxygen species (ROS), e.g., as by-products of an impaired mitochondrial respiration (Sohal, 1991). Increased generation of ROS will further damage neighboring mitochondrial protein complexes and enzymes, as well as membranes and DNA, and this in turn might further accelerate the aging process.

Several of the well-known negative consequences of solar radiation are mediated by ultraviolet (UV)-induced generation of ROS (Scharffetter-Kochanek *et al*, 1997). Increasing levels of ROS lead to an enhanced redox signalling and finally to the activation of specific target genes like matrixmetalloproteinases, which in turn degrade dermal collagen bundles and thus contribute to the formation of facial wrinkles (Fisher *et al*, 1997). Physiologically, the cellular level of ROS can be balanced and counteracted by an effective endogenous antioxidant system (Sies, 1985).

Oxidative damage of cellular and extracellular components activates intrinsic repair mechanisms, which necessarily require ATP for full functionality. The PCr (phosphocreatine)/CK (creatine kinase) system together with the recently discovered epidermal creatine transporter (CRT) (Schlattner *et al*, 2002) provide human skin with an important tool to cope efficiently with conditions of high-energy demand. The free energy of ATP is stored and transported in the form of PCr from sub-cellular sites of energy production, e.g., mitochondria, to places of high-energy requirements, where CK activity can replenish cellular ATP *in situ* (Bessman and Carpenter, 1985; Wallimann *et al*, 1992, 1998). Predominant isoforms of CK consist of cytosolic MM-CK (muscle-type), mainly found in muscle cells, as well as cytosolic BB-CK (brain-type) and ubiquitous mitochondrial Mi-CK, with the last two mainly located in the brain but also present in skin (Schlattner *et al*, 2002). The importance of creatine and the CK system for normal cell function has been elucidated in transgenic mice lacking the expression of CK (Steeghs *et al*, 1997; Gorselink *et al*, 2001; Crozatier *et al*, 2002; Janssen *et al*, 2003). These animals showed muscular and neurological dysfunctions and

Abbreviations: BB-CK, cytosolic brain-type (ubiquitous) CK; CK, creatine kinase; CRT, creatine transporter; EM, emission; EX, excitation; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazolcarbocyanine iodide; K(m), Michelis–Menten constant; Mi-CK, mitochondrial CK; MM-CK, cytosolic muscle-type CK; MMP, mitochondrial membrane potential; PBS, phosphate-buffered saline; PCr, phosphocreatine; ROS, free reactive oxygen species; TCA, trichloroacetic acid; UVA, ultraviolet A (320–400 nm)

phenotypes that have some similarities with the clinical symptoms of humans suffering from the so-called "creatine deficiency syndrome" (Leuzzi, 2002).

Cells attain their physiological levels of creatine, either by biosynthesis from the amino acids arginine, glycine, and methionine in the kidney, liver, and pancreas of vertebrates including humans (Walker, 1979), and/or alternatively by ingestion of meat and fish (Balsom *et al*, 1994). Creatine is transported via the blood circulation and is taken up into cells by a Na^+- and Cl^--dependent CRT protein (Snow and Murphy, 2001; Speer *et al*, 2004). Once inside a cell, creatine can be stored at high concentrations (e.g., 40 mM for muscle cells). In addition to this, creatine is well known for its protective effects in the treatment of neurological and atherosclerotic diseases (Wyss and Schulze, 2002), as well as traumatic brain and nerve injury (Sullivan *et al*, 2000; Hausmann *et al*, 2002). Furthermore, there are first indications that creatine supplementation results in less UV-induced mitochondrial DNA mutations in skin cells (Krutmann, 2001).

Despite the important function of creatine as an essential energy precursor, the endogenous synthesis of creatine in humans is not sufficient and creatine has to be supplemented by daily food intake, especially fish and meat. Moreover, cutaneous cells may show signs of a declining creatine level, probably caused by a stress and age-related decline of dermal vascularization (Chung *et al*, 2002).

So far little is known about the question of whether oxidative stress affects the CK system and/or creatine metabolism in healthy skin. But since there is a close relationship of free radical reactions and energy metabolism (Nohl *et al*, 1997) and since CK, especially Mi-CK, is a prime target for oxidative damage (Stachowiak *et al*, 1998; Dolder *et al*, 2001; Wendt *et al*, 2003), we investigated, however, the functional importance of creatine and the CK system in human skin and its modulation by creatine supplementation with a focus on oxidative stress conditions. Here, we present results that creatine has marked protective effects against oxidative stress on keratinocytes *in vitro* and against UV-induced damage *in vivo*.

Results

CRT and CK in human skin The localization of CRT and CK isoenzymes in human skin was examined by immunohistochemistry. Cryosections were stained with isoenzyme-specific anti-CK, as well as with anti-CRT antibodies. Figure 1*A* shows the localization of the soluble BB-CK isoform in the epidermis of human skin. BB-CK is mainly located in the cytosolic compartment of all epidermal layers (EL). In contrast, the dermis showed only little staining. Both controls without primary antibody (Fig 1*G*) as well as immunohistochemistry using an antibody against MM-CK (data not shown) showed no signal in the epidermal skin layer. These results are consistent with those published earlier (Schlattner *et al*, 2002). Immunohistochemistry against uMi-CK also showed significant staining of the epidermal layer of the human skin (Fig 1*C*). Anti-CRT antibodies reacted preferentially with cell membranes of cells in the EL. Indirect immunofluorescence using the polyclonal anti-human CRT antibody

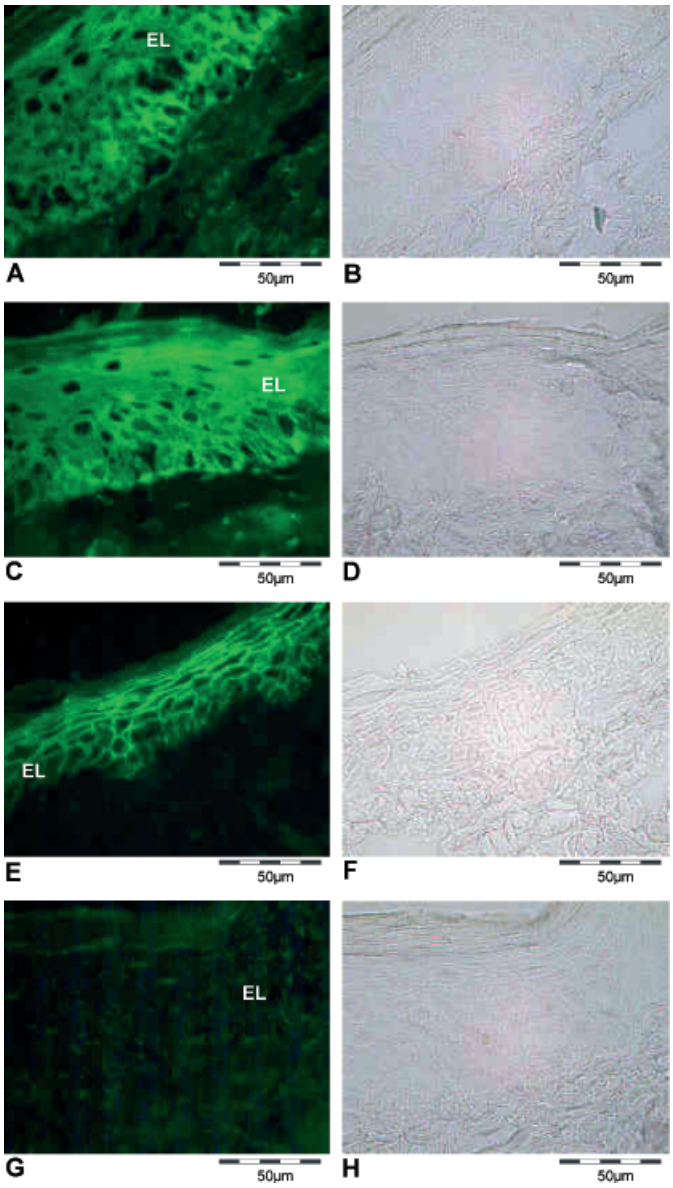

Figure 1
Immunohistochemical localization of creatine kinase (CK) and creatine transporter (CRT) in human skin. Phase contrast (*B*, *D*, *F*, *H*) and immunofluorescence microscopy images (*A*, *C*, *E*, *G*) of 7 μM cryosections of healthy human skin are shown. Sections were stained for BB-CK (cytosolic brain-type (ubiquitous) CK) (*A*), Mi-CK (mitochondrial CK) (*C*), and CRT (*E*) with appropriate negative controls (*G*, *H*). Goat-anti-rabbit IgG AlexaFluor488-conjugate was used as secondary antibody (*scale bars* = 50 μm).

(Fig 1*E*) revealed a distinct staining in the plasma membrane of keratinocytes in the entire epidermis, whereas no staining was found in the negative control (Fig 1*G*).

Strong BB-CK (Fig 2*A*) and uMi-CK (Fig 2*C*) staining was also detectable in immunohistology of cell cultures using primary human keratinocytes of healthy donors. In these cells *in vitro*, BB-CK was mainly located in the cytosolic compartment, but was also clustered in a particular pattern around the nucleus. uMi-CK showed a more clustered pattern, typical for mitochondria, as could be proven by colocalization with an antibody against a mitochondrial protein (Biotrend, Cologne, Germany). The negative control remained unlabelled (Fig 2*E*).

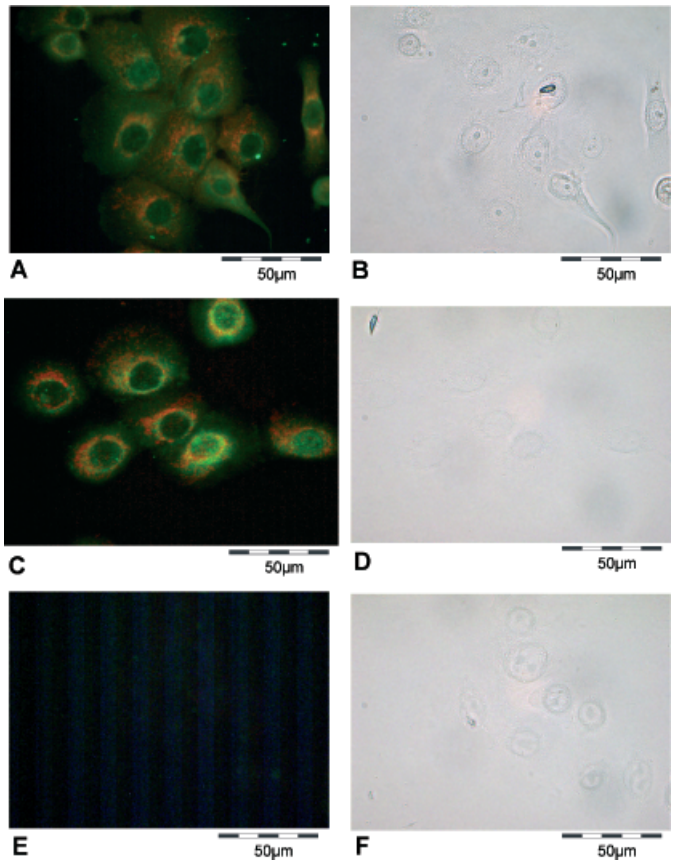

Figure 2
Immunohistochemical localization of creatine kinase (CK) in human primary keratinocytes. Primary human keratinocytes obtained from healthy donors were double stained for BB-CK (cytosolic brain-type (ubiquitous) CK) (green fluorescence) and mitochondria (red fluorescence) (*A*) following methanol–acetone fixation with appropriate negative control (*E*). Further keratinocytes were stained for both Mi-CK (mitochondrial CK) (green fluorescence) and mitochondria (red fluorescence) (*C*) following methanol–acetone fixation. *B*, *D*, and *F* show phase contrast images. Goat-anti-rabbit IgG AlexaFluor488- and goat-anti-mouse IgG AlexaFluor546-conjugate were used as secondary antibody (*scale bars* = 50 µm).

Human epidermal volar forearm skin was isolated by the suction blister technique from the untreated control site and from both the placebo-treated and sites treated with a 0.1% creatine-containing formulation for 1 wk, respectively. Figure 3 shows a representative western blot analysis of CK isoenzymes and CRT taken from suction blister epidermis cells of the appropriate skin site (Fig 3*A*) in comparison with proteins from human primary keratinocytes *in vitro* (Fig 3*B* and *C*). For CRT, a distinct band with an apparent molecular weight of 70 kDa was found (Fig 3*A*). The detectable staining intensity of CRT in blister epidermis was, however, not affected by any of the treatments, e.g., ± creatine (Fig 3*A*). Samples of cellular extracts from identical suction blister epidermis showed no staining using an antibody against MM-CK (data not shown). In contrast, for BB-CK, a clear signal of about 45 kDa was detectable in suction blisters as well as in human primary keratinocytes (Fig 3*B*). Mi-CK (about 40 kDa) was also detected in cells from suction blisters and in human primary keratinocytes (Fig 3*C*).

Furthermore, the cellular uptake of creatine was measured using ^{14}C-labelled creatine (Fig 4). A strong increase of ^{14}C-creatine inside keratinocytes can be observed within

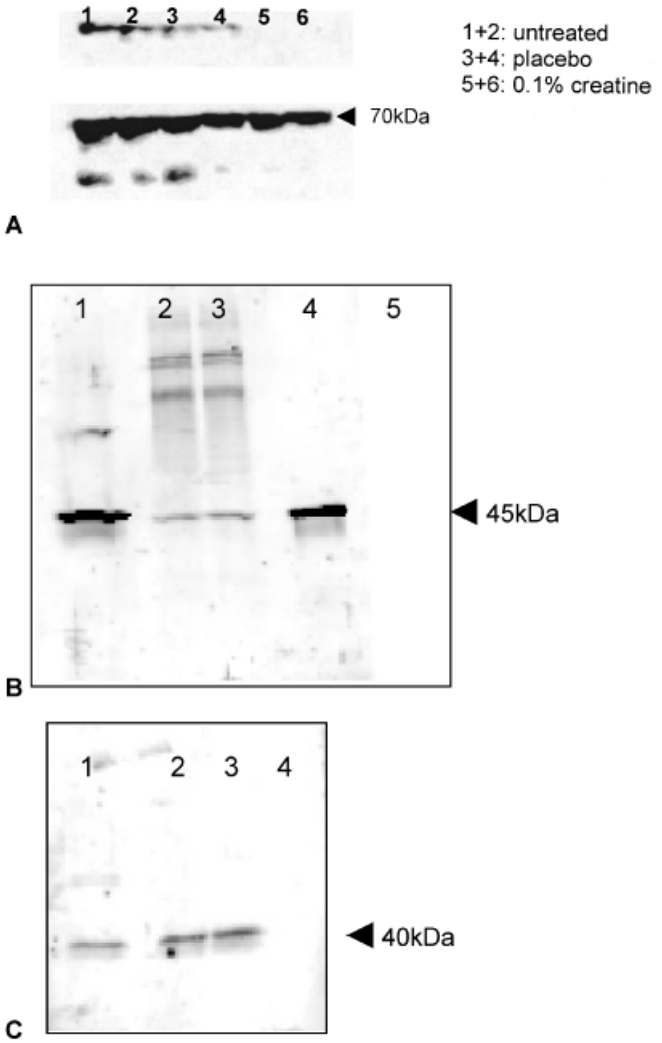

Figure 3
Expression of creatine transporter (CRT) and creatine kinase (CK) isoforms BB (brain-type (ubiquitous)) and Mi (mitochondrial) in suction blister epidermis and primary keratinocytes. Western blot analysis of CRT (*A*), BB-CK (*B*), and Mi-CK (*C*). Human suction blister epidermis of untreated (*lanes 1*, *2*), creatine- (*lanes 5*, *6*), and placebo-treated skin sites (*lanes 3*, *4*) were isolated and separated by SDS-PAGE. In western blot analysis, 10 µg of total protein was loaded per lane as indicated and CRT was detected (*A*). ◀ indicates a molecular weight of about 70 kDa for CRT. Further human suction blister epidermis of an untreated skin site (*lane 1* in *B* and *C*) and cell lysates (*lanes 2, 3* in *B* and *C*) were separated by SDS-PAGE. In western blot analysis, 15 µg (for the anti-Mi-CK blots, *C*) or 20 µg (for the anti-BB-CK blots, *B*) of total protein was loaded per lane as indicated and BB-CK (*B*) and Mi-CK (*C*) were detected. Isolated BB-CK (*lane 4*) served as a positive control in *B*. As negative control no primary antibody was used (*lane 5* in *B* and *lane 4* in *C*, negative). ◀ indicates a molecular weight of about 45kDa for BB-CK (*B*) and 40 kDA for Mi-CK (*C*). Molecular weight standards were used from Bio-Rad (Munich, Germany; *A*: Kaleidoscope, *B* and *C*: Precision Plus, dual color). All western blots were repeated three to five times and representative blots were chosen as figures.

60 min. The addition of 2 mM guanidionopropionic acid (GPA) after 20 min, a competitive inhibitor of the CRT, significantly reduced creatine uptake.

CK enzymatic activity studies The activity of total CK was examined in extracts of different cell types of human skin (Table I). Analysis of specific CK activity in extracts of human dermal fibroblasts contrasted to the relatively high CK

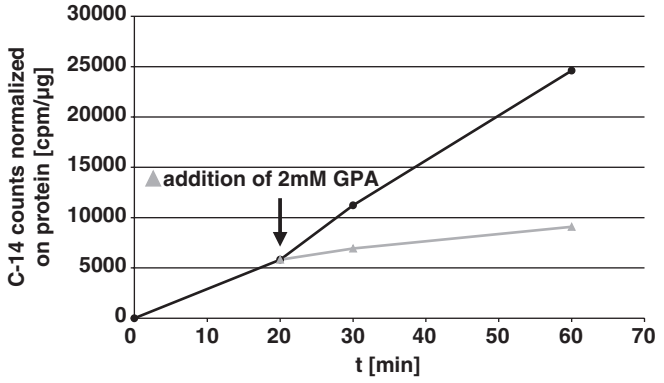

Figure 4
Cellular uptake of creatine. The uptake was measured by addition of ^{14}C-labelled creatine (●) to human primary keratinocytes. At the time indicated by the arrow, 2 mM guanidinoproionic acid (GPA), a competitive inhibitor of creatine uptake, was added (▲).

activity in human keratinocytes, both obtained from healthy donors. These data correlated with histological findings showing a lower CK expression in human dermal cells (Fig 1) in comparison with the relatively high expression (Fig 2*A*) found in human keratinocytes. The fluid extracted from suction blisters also showed some CK activity (Paavonen *et al*, 1988). All data were normalized relative to the total amount of proteins.

To investigate age-related differences, CK activity of epidermal skin cells obtained from young (not older than 35 y) and old human donors (not younger than 65 y) were analyzed. We observed a substantial but not significant decline of CK activity in cells obtained from old donors (n = 10), reduced to 84% (± 26%) in comparison with the activity in cells from young donors (n = 10), which were set to be 100% (± 23%) (data not shown).

Modulation of CK activity and cellular uptake of exogenous creatine To evaluate the impact of creatine supplementation, the activity of BB-CK was determined following the incubation of keratinocytes with creatine in comparison with untreated control keratinocytes (Fig 5). Pre-loading of old donor cells with 150 µM creatine for 24 h resulted in a statistically significant increase (+23%) in specific CK activity in comparison with untreated cells (100%). The protection of CK in young donor cells following preloading with 150 µM creatine was only marginal (Fig 5).

Mi-CK seemed to have a higher specific activity compared with cytosolic CK and was further stimulated by creatine preloading (Table II). In order to exclude BB-CK contamination in the organelle fractions, western blot analysis was carried out, with both of the cytosolic and the organelle fraction. BB-CK could only be detected in the cytosolic fraction, whereas Mi-CK was only found in the organelle fraction (data not shown).

We also obtained evidence that creatine predominantly protected Mi-CK against H_2O_2 stress conditions (Fig 6*A*). H_2O_2, even at small concentrations of 1 and 55 µM (as direct oxidative stress), caused a significant decrease in the CK activity of about 40% and 50% (*open bars*), respectively, compared with the untreated control (100%). Pre-treatment of keratinocytes with 150 µM creatine (*closed bars*) prior to H_2O_2 incubation improved CK activity by 10% (at 1 µM H_2O_2) or 19% (at 55 µM H_2O_2) when compared with untreated control, indicating a protection of the enzyme against oxidative damage by creatine.

Table I. CK activity in human skin cells

	Fibroblasts	Suction blister fluid	Keratinocytes
Creatine kinase activity/protein (EU)	0.9 ± 0.17	0.7 ± 0.25	7.3 ± 1.71

Cytosolic extracts of early passaged cultivated dermal fibroblasts (n = 6), suction blister fluid (n = 6) and primary human keratinocytes (n = 17) obtained from healthy donors were analyzed in a CK activity assay. Data represent the mean ± SD.
CK, creatine kinase; EU, µmol PCr (phosphocreatine) per s per µg protein at 25°C.

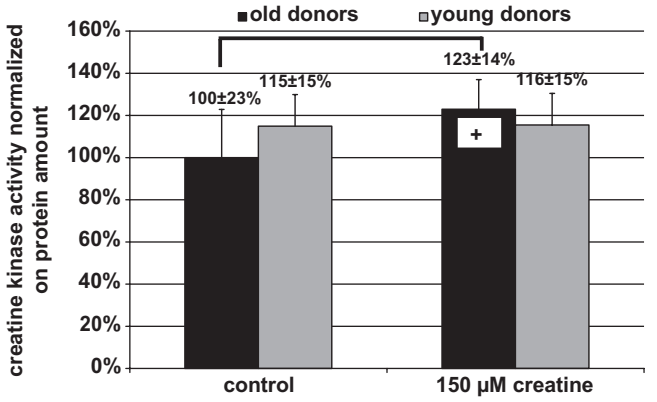

Figure 5
Creatine supplementation and creatine kinase (CK) activity in human keratinocytes. Cytosolic extracts of early passaged cultivated primary human keratinocytes obtained from old (n = 13; not younger than 65 y) and young donors (n = 8; not older than 35 y) were incubated with 150 µM creatine for 24 h in culture medium and subsequently investigated for CK activity (+, significant difference; $p < 0.05$). Error bars indicate standard deviation (SD).

H_2O_2 concentrations were chosen according to our own pilot experiments and literature data. Our cell vitality tests with dermal fibroblasts showed that H_2O_2 concentrations higher than 100 µM might lethally shock skin cells, in particular the older the donors are (data not shown). In addition, we were interested in a senescent phenotype that was observed by different studies in human fibroblasts applying H_2O_2 in the low micromolar range (Chen and Ames, 1994; Bladier *et al*, 1997; Chen *et al*, 1998). Thus, the concentration range of 1–55 µM H_2O_2 that we have chosen for our study should resemble levels of oxidative stress that could occur during the human aging processes.

We examined no antioxidative properties of creatine in concentrations up to 450 µM in a cell-free assay (Fig 6*B*). The assay performed estimates the radical scavenging capacity of creatine and vitamin C served as a positive control.

Cellular responses in human keratinocytes following UVA-induced oxidative stress and protection by creatine The formation of intracellular soluble peroxides in keratinocytes from old donors (n = 9) following UVA radiation is demonstrated in Fig 7*A*. Using doses of UVA radiation of 5, 10, and 20 J per cm^2, the level of intracellular peroxides measured by dichlorodihydrofluorescein (DCHF)

Table II. CK activity in cytosolic and organelle fractions with and without creatine incubation

	Cytosol	Cytosol + 150 μM creatine	Organelles	Organelles + 150 μM creatine
Creatine kinase activity/ protein (EU)	1.1 ± 1.5	1.2 ± 1.5	5.7 ± 1.7	7.0 ± 2.5

Primary human keratinocytes obtained from healthy old donors (n = 3) were loaded with creatine (150 μM for 24 h) in comparison to untreated control cells before organelle separation. Afterwards CK activity of the cytosolic and organelle containing fraction was investigated. Data represent the mean ± SD.

CK, creatine kinase; EU, μmol PCr (phosphocreatine) per s per μg protein at 25°C.

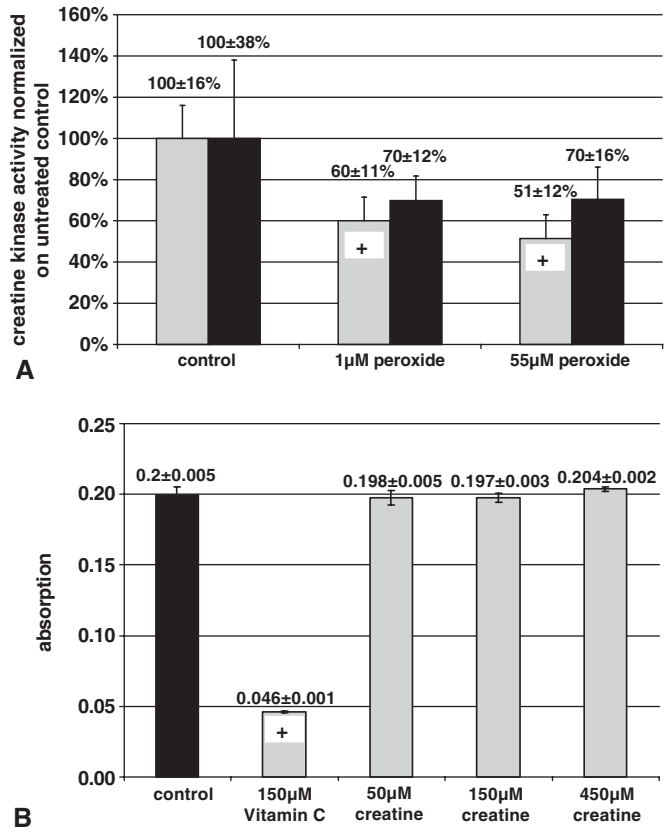

Figure 6

Inhibition of creatine kinase (CK) activity of human keratinocytes by H_2O_2 and protection by exogenous creatine. Measurement of CK activity (*A*) of cytosolic extracts of early passaged cultivated primary human keratinocytes obtained from healthy donors (n = 3) using different low doses of H_2O_2 as mild oxidative stress (*open bars*). Preloading of appropriate keratinocytes with creatine (150 μM for 24 h) prior to H_2O_2 stress is shown in *closed bars*. Data represent the mean ± SD and were normalized on unstressed control levels (= 100%). No antioxidative properties (*B*) were observed for creatine concentrations up to 450 μM, compared with vitamin C as a positive control (all data n = 3) (+, significant difference; $p < 0.05$).

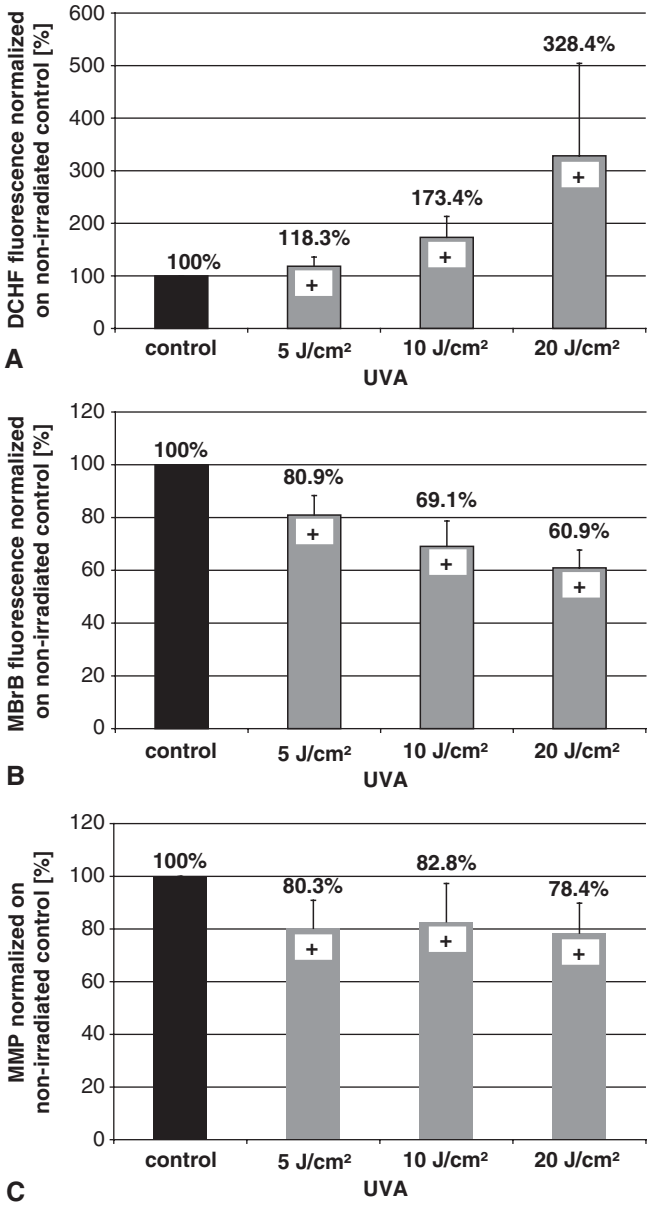

Figure 7

Modulation in cellular mechanisms of human keratinocytes following ultraviolet A (UVA) irradiation. Three different physiological parameters were analyzed in early passaged cultivated primary human keratinocytes obtained from healthy old donors following different doses of UVA irradiation (5, 10, and 20 J per cm^2). The formation of soluble peroxides (*A*) was measured by dichlorodihydrofluorescein (DCHF) fluorescence in cells from nine healthy donors. Measurement of intracellular thiols (*B*) in cells from seven donors used monobromobimane as fluorescent dye. Mitochondrial membrane potential (MMP) in keratinocytes from ten donors was detected by evaluation of 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazol-carbocyanine iodide (JC-1) fluorescence (*C*). All fluorescent data represent the mean ± SD and were related to appropriate cell number and qualified on non-irradiated control (= 100%) (+, significant difference; $p < 0.05$).

fluorescence increased significantly in a concentration-dependent manner to values up to 328%, respectively, compared with the non-irradiated control cells (Fig 7*A*). Monobromobimane (MBrB) fluorescence as an indicator of intracellular thiol levels (Fig 7*B*) revealed a dose dependent significant decline in total cellular thiol groups after UVA radiation (5, 10, and 20 J per cm^2) with keratinocytes from old donors (n = 7) reaching values as low as 61%, compared with non-irradiated control cells (100%). With regard to UV-induced damage in mitochondrial energy supply, mitochondrial membrane potential (MMP) (JC1-fluorescence) of control cells (non-irradiated keratinocytes) and UVA-irradiated keratinocytes from old (not younger than

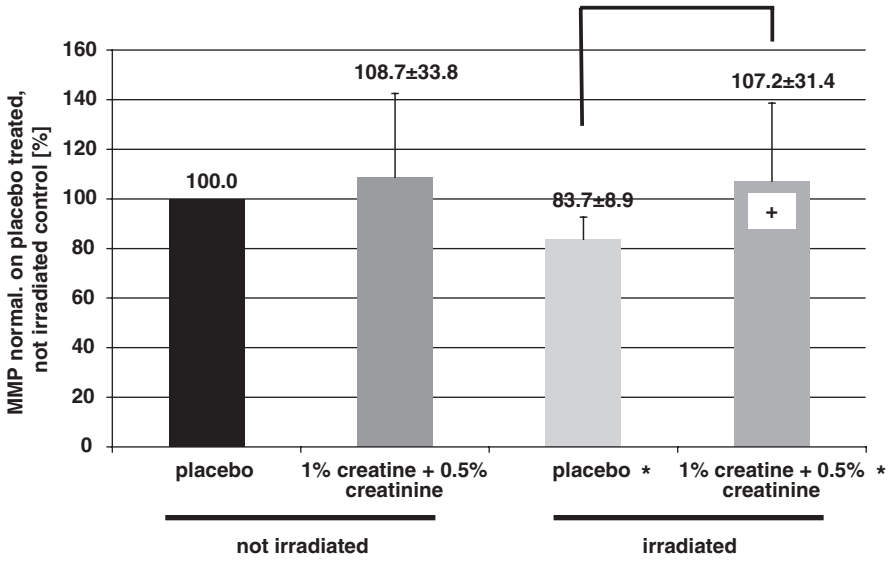

Figure 8
Mitochondrial membrane potential (MMP) protection following topical application of creatine/creatinine. Healthy old volunteers ($n = 8$; average age of 65.2 y) topically applied a 1% creatine/0.5% creatinine-containing formulation twice a day for a period of 4 wk. Human suction blister epidermis of placebo- and verum-treated skin sites was isolated and epidermal cells separated for fluorescent evaluation of MMP. All fluorescent data represent the mean $\pm$ SD and were related to appropriate cell number and to placebo-treated, non-irradiated control ($= 100\%$). The two bars on the left represent non-irradiated cells; the two bars on the right represent ultraviolet (UV)-irradiated cells (* indicates irradiation; irradiation with 15 J per cm^2 once after cell isolation) ($+$, significant difference; $p < 0.05$).

65 y) donors ($n = 10$) were evaluated as shown in Fig 7*C*. The MMP decreased significantly following irradiation with 5 J per cm^2 by up to 80.3% relative to the non-irradiated control. UVA irradiation with higher doses (10 and 20 J per cm^2) led to comparable reduction of MMP values.

On the basis of several promising *in vitro* results (data not shown) concerning the protective effect of creatine on the MMP, we performed *ex vivo* studies on human suction blister epidermis to evaluate the protective effects of a topical creatine/creatinine application in stabilizing mitochondrial function.

Healthy old volunteers (average age of 65.2 y) topically applied a stabilized creatine formulation (1% creatine/0.5% creatinine) twice a day for 4 wk on their upper arm. Afterwards, epidermal cells were isolated via suction blister and irradiated to examine MMP (Fig 8). In response to the irradiation with 15 J per cm^2 UVA light, epidermal cells from placebo-treated skin sites showed a decline in their MMP by 16.3% compared with non-irradiated control cells. In contrast, cells from the creatine/creatinine-treated skin sites showed a statistically significant maintenance of their MMP, even after irradiation compared with the irradiated placebo control cells (Fig 8).

Discussion

During daily human life, the skin is particularly exposed to different environmental stressors, which can more or less influence the physiological behavior of cutaneous cells and thus the skin as an entire organ. Any disturbance in the cellular homeostasis is usually counteracted by various intrinsic mechanisms, aiming to rebalance the deregulated physiological processes rapidly. For this purpose, each single cell is provided with several repair mechanisms, depending mostly on a sufficient energy supply to the appropriate cell and tissue. For various cells and organs, it has been shown that changes in the cellular energy state occur during aging, especially in muscle tissue (Carmeli *et al*, 2002). Krebs cycle enzymes in skeletal muscle of old rats (Ermini, 1976), e.g., as well as important compounds for cellular energetics, such as citrate, PCr, and ATP in aged human skeletal muscles (Pastoris *et al*, 2000), are reported to decline notably.

This study shows the significance of the PCr/CK system for epidermal energy supply and the beneficial effects of creatine supplementation both *in vitro* and *in vivo*.

We found that both creatine kinase subtypes, BB-CK and Mi-CK, as well as CRT, are expressed in human skin, showing high levels in the epidermis but less in the dermis (Fig 1). This confirms a recent publication from Schlattner *et al* (2002) on BB-CK in murine and human cells. Regarding the localization in the epidermis, there was no gradient of CRT, BB-CK, or Mi-CK detectable. These data clearly demonstrate that human skin contains a functional PCr/CK system.

We measured total CK activities of dermal fibroblasts, keratinocytes, and suction blister fluids obtained from donors of different ages. In accordance with histological findings, the highest enzyme activity was found in keratinocytes (Table I), again indicating an increased energy turnover in epidermal cells. The lower BB-CK activity in dermal fibroblasts (Fig 1 and Table I) is likely due to a lower expression of this enzyme in these cells *in vivo* (Fig 1).

Furthermore, we observed a lower total CK activity in epidermal skin cells obtained from old donors. These data correlate with results from different experiments using skeletal muscle and heart muscle, clearly showing that both PCr amount (Verzar and Ermini, 1970; McCully *et al*, 1991) and CK activity decrease with age (Steinhagen-Thiessen and Hilz, 1976; Bogatskaia and Shegera, 1981). This decline in skin CK activity may be caused by the generation of ROS during cutaneous aging (Harman, 1956; Dolder *et al*, 2001). This is supported by the fact that CK, specifically Mi-CK, is a primary target for ROS, especially peroxynitrite (Stachowiak *et al*, 1998). It is fully in line with the observed substantial inactivation of CK after application of low concentrations of H_2O_2 (Fig 6*A*). Oxidative stress and free radical reactions in skin cells evolve during inflammation or as by-products of mitochondrial respiration, especially during aging (Sohal, 1991; Lee, 1997). Enhanced stress conditions in turn may cause further damages in mitochondria

and can reduce cellular energy capacity, as evaluated by parameters like MMP (Fig 7*C*). With regard to the sensitivity of CK activity towards oxidative stress, energetic deficiencies in old keratinocytes might be caused by a loss of Mi-CK activity, which seems of major importance in keratinocytes (Table II). It is known from the literature (Ronquist *et al*, 2003) and our own experiments (data not shown) that there are only slight differences in mitochondrial number between keratinocytes and dermal fibroblasts; however, particularly keratinocytes contain more structurally abnormal mitochondria. It can be speculated that this change in mitochondrial morphology and function may be partially caused by the limited availability of substrates like creatine and a resulting destabilization of octameric Mi-CK (Table II). Any lack in its function may directly affect energy supply in the skin and further accelerate cutaneous aging.

In order to investigate oxidative stress reactions, we irradiated human skin cells from old donors using different doses of UVA light, known to induce the formation of ROS (Scharffetter-Kochanek *et al*, 1997). Even low doses of 5 J per cm^2 UVA substantially elevated cellular peroxide levels (Fig 7*A*). This effect was accompanied by both a decrease in the level of intracellular thiols (Niggli and Applegate, 1997), one main parameter of the non-enzymatic antioxidant system, as well as a decline in the MMP, indicating ROS-mediated damage to mitochondrial energy supply (Fig 7*B*, *C*).

We finally demonstrated the positive effects of creatine supplementation. Obviously, human keratinocytes are able to take up creatine (Fig 4). This accumulation of supplemented creatine in keratinocytes leads to a significant increase in total CK activity (Fig 5) and substantially stabilizes and protects CK activity *in vitro* even under conditions of direct oxidative stress (Fig 6*A*). Our data imply a correlation between the observed substantial decline of total CK activity in epidermal cells and of oxidative stress conditions, comparable to those that occur during aging. It can also be speculated that the intrinsic level of epidermal creatine declines as a function of age mainly caused by a lack of vascularization (Chung *et al*, 2002). This conclusion seems to be justified, because elevating cellular uptake of creatine, as a substrate for CK, reversed the age-related loss in CK enzyme activity (Fig 6*A*). This may be due to a protection of the enzymes, both BB-CK and Mi-CK, by creatine against oxygen-radical damage (Lawler *et al*, 2002) and we have strong evidence that this effect is not caused by any antioxidative property of creatine itself (Fig 6*B*). Any antioxidative or other indirect effects of creatine mostly occur when creatine is applied in high concentrations, because it may additionally influence the availability of methyl groups for protein synthesis by sparing *S*-adenosyl-methionine (Stead *et al*, 2001). In our experiments using a low concentration of creatine (150 μM), these antioxidative effects are negligible and one is likely looking at direct effects of creatine as an energy precursor for keratinocytes. At the chosen concentration of creatine, CRT is well saturated (K(m) (Michaelis–Menten constant) of CRT for creatine is in the range of approximately 20–30 μM) (Dodd and Christie, 2001; Peral *et al*, 2002) and thus skin cells can rapidly accumulate creatine. This cellular uptake of creatine facilitated by the creatine transporter and the enrichment of applied creatine in human skin cells (Fig 4) can also explain the effects of creatine on the MMP (Fig 8; Daly and Seifter, 1980). Generally, CK is specifically located at places of energy demand and energy production and plays an important role in the energetics of Ca^{2+}-homeostasis and mitochondrial membrane stability (Wallimann *et al*, 1998). Thus, the protective effect on mitochondria may be explained by the stabilization of Mi-CK by creatine, even under conditions of oxidative stress (Fig 6*A*). Mi-CK is a cuboidal-shaped octamer that stabilizes and cross-links cristae- or inner/outer membranes to form parallel membrane stacks. Mi-CK octamers and creatine have been reported to modulate mitochondrial permeability transition pore opening (Wallimann *et al*, 1998; Dolder *et al*, 2003) in some organs. These findings corroborate with data by Krutmann and colleagues, clearly showing the protective role of creatine treatment on the formation of mitochondrial common deletion mutation in skin cells (Krutmann, 2001). The common deletion mutation is well known to be closely related to mitochondrial decay in dermal aging as well. With increasing age, fibroblasts switch from a matrix-producing to a matrix-degrading phenotype (Campisi, 1998). One possible explanation may be a decline in cellular energy supply (Hagen *et al*, 1997; Rottenberg and Wu, 1997). In fact, we have preliminary evidence that stabilizing mitochondrial energetic capacity by creatine application *in vitro* and *in vivo* can substantially restimulate synthesis of dermal matrix components like collagen (data not shown).

Our data indicate that the epidermal creatine system, which is very important for cellular energy metabolism, obviously declines under oxidative stress conditions, including skin aging processes. Application of creatine to skin cells *in vitro* and *in vivo* can recharge these cells energetically, and thus protect them against free radical-induced cell damage. We are currently investigating further effects of a topical creatine application using cosmetic and dermatological formulations, especially for prophylaxis and treatment of aging human skin.

Material and Methods

Cell culture Human skin biopsies were isolated from healthy old (not younger than 65 y) and young (not older than 35 y) donors. Primary human dermal cells were enzymatically prepared using a standardized dispase (Boehringer Mannheim, Mannheim, Germany) digestion technique. The dermal fraction was cultured at 37°C and 7% CO_2 (in air) in six-well plates containing Dulbecco's modified Eagle's medium (Life Technologies, Eggenstein, Germany) supplemented with 10% fetal calf serum (Life Technologies) and penicillin/streptomycin (50 IU per mL; 50 μg per mL, Life Technologies). After 5–6 wk of incubation, confluently grown fibroblasts were seeded into 96-well microtiter plates (Greiner, Frickenhausen, Germany). Human primary keratinocytes were isolated from the epidermis through trypsin digestion (PAA, Linz, Austria) and cultured in KGM-2 (Cambrex, Apen, Germany). After cells reached a confluency of 50%–70%, keratinocytes were split 1:2. After 2–3 d of incubation, cells were used for the experiments described below.

Suction blister Epidermal roofs were harvested from suction blisters of human skin (Kiistala, 1968). For blistering on an area of about 5 mm in diameter, a vacuum of 300 mbar was used for about 2 h. Primary skin cells were then harvested by trypsin digestion from the freshly prepared epidermal biopsies.

UVA source UVA irradiation (5, 10, 15, and 20 J per cm^2 for one time) was performed using a Dermalight 2020 illuminator (Dr Hönle, Planegg, Germany). The emitted dose was quantitated using a UV-radiometer IL1700 (International Light, Newburyport, Massachusetts).

Evaluation of MMP The MMP was measured using the dye 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazol-carbocyanine iodide (JC-1) (Molecular Probes, Eugene, Oregon), which can be used to specifically quantify alterations in MMP in living cells (Cossarizza *et al*, 1993). Fluorescence was measured spectrometrically (EX 485 nm/EM 530 nm and EM 485 nm/EM 590 nm; Cytofluor II, Perseptive Biosystems, Freiburg, Germany). All data presented were background corrected by measuring fluorescent values without cells.

Evaluation of antioxidative properties (ABTS assay) Antioxidative properties of creatine in comparison with vitamin C were investigated as described in the literature by Re *et al* (1999).

Determination of soluble peroxides Intracellular peroxides were detected spectrometrically in a Cytofluor II (Perseptive Biosystems) using the probe Dichlorodihydrofluoresceindiacetat (DCHF-DA), which specifically reacts with peroxides (Rosenkranz *et al*, 1992; Suzuki *et al*, 1997). The quantification of specific fluorescence (EX 485 nm/EM 530 nm) in relation to cell number was performed in 96-well microtiter plates also using the Cytofluor II. All data presented were background corrected by measuring fluorescent values without cells.

Evaluation of intracellular thiol groups Intracellular thiol groups were measured spectrometrically in a Cytofluor II (Perseptive Biosystems) using the probe MBrB, which specifically reacts with thiol groups (Durand and Olive, 1983; Cotgreave *et al*, 1988). The quantification of the specific fluorescence (EX 390 nm/EM 460 nm) in relation to cell number was performed in 96-well microtiter plates using the Cytofluor II. All data presented were background corrected by measuring fluorescent values without cells.

Incubation with creatine Creatine (Goldschmidt, Essen, Germany; SKW Trostberg, Freising-Weihenstephan, Germany; Sigma, Munich, Germany) was added at a final concentration of 150 μM to subconfluently grown (about 70% confluency) keratinocytes. 150 μM creatine is slightly above normal serum concentrations (30–100 μM) (Vanakoski *et al*, 1998; Schedel *et al*, 1999). Creatine was prepared in KGM-2, filtered, and then added to the culture medium. After 24 h incubation cells were washed twice with phosphate-buffered saline (PBS) and used for further experiments.

Uptake of ^{14}C-creatine ^{14}C-creatine (Biotrend; activity of 1 mCi per mL) was added to human primary keratinocytes in six-well plates, following washing once with PBS. GPA (Sigma) was used as a competitive inhibitor for CRT at a concentration of 2 mM and was added after 20 min of incubation with ^{14}C-creatine. Following incubation, cells were washed three times with PBS and cytoplasmatic extracts were measured in a liquid scintillation counter (Pharmacia, Freiburg, Germany) and ^{14}C-counts were normalized on total protein amount (μg).

Topical application of creatine Volunteers (average age of 65.2 y) topically applied a formulation containing either 0.1% creatine for western blot-analysis or 1% creatine/0.5% creatinine (Goldschmidt, SKW Trostberg) *versus* a placebo formulation without creatine and creatinine twice a day for a period of 1 or 4 wk, respectively. Creatinine was added to the formulation to maintain a chemically balanced quantity of highly reactive creatine, which, if not stabilized, readily undergoes rearrangement to creatinine in aqueous systems. The study was conducted according to the Declaration of Helsinki Principles and participants gave their written informed consent. All experiments were performed and approved by the R&D, Beiersdorf AG, Hamburg, Germany.

Antibodies and immunoblotting Proteins from cell extracts (10 μg per lane) were separated by polyacrylamide gel electrophoresis under denaturing conditions on 10% SDS-PAGE gels. Subsequently, the proteins were electrotransferred onto nitrocellulose or polyvinylidine fluoride membranes and, after blocking with 10% milk powder, incubated with primary anti-BB, anti-MM, or anti-Mi-CK antibodies, as well as with anti-creatine-transporter antibodies (anti-human anti-BB-CK, anti-MM-CK, and anti-CRT antibodies from Biotrend (Köln, Germany), anti-human Mi-CK described in Schlattner *et al*, 2002), followed by secondary antibodies (anti-rabbit IgG, Sigma), the latter conjugated to horseradish peroxidase (HRP). The protein bands, blotted on nitrocellulose or PVDF sheets, were visualized using the Lumilight Plus Western Blot Detection Kit (Roche, Mannheim, Germany).

Immunohistology Samples of human skin were embedded in TissueTek (Leica, Nussloch, Germany), frozen in liquid nitrogen, and cut with a microtome into 5–7 μm thin sections. After fixation in methanol/acetone (1:1) at −20°C for 10 min, the sections were washed three times with PBS and blocked in 1% bovine serum albumin (BSA). Sections were then incubated overnight at 4°C in a moist chamber with primary antibodies against either BB-CK, Mi-CK, or CRT diluted in PBS containing 0.1% BSA. All polyclonal antibodies (BB-CK and CRT: Biotrend, Mi-CK: Schlattner *et al*, 2002) were diluted 1:1000. For colocalization a monoclonal mouse-anti-human antibody against a 60 kDa mitochondrial antigen (Biotrend) was used in a 1:1000 dilution. After washing three times in PBS, sections were incubated with secondary antibodies (AlexaFluor 488 goat-anti-rabbit IgG or AlexaFluor 546 goat-anti-mouse IgG, Molecular Probes, Eugene, Oregon) diluted 1:1000 for 1 h at room temperature in a moist chamber. Sections were washed three times in PBS and mounted in Mowiol (Calbiochem, San Diego, California). Fluorescence images of sections were recorded on a fluorescence microscope (Carl Zeiss, Oberkochen, Germany) with an attached closed-circuit display (CCD) camera.

CK activity assay Cytoplasmic extracts (10 μL) of creatine-treated and untreated control cells were used in a creatine kinase assay (Sigma). 50 μL creatine solution was added to one sample of each cytoplasmic extract (test), and 50 μL tris(hydroxymethyl) Methanamine (TRIZMA) buffer was added to another sample (blank). After addition of 50 μL water and 5 min incubation at 37°C, 4 μL ATP-glutathione was added and the samples were incubated at 37°C for 1 h. The reaction was then stopped with 80 μL 20% tricchloroacetic acid (TCA) and filtered through a multiscreen plate (Millipore, Billerica, Massachusetts). After addition of 200 μL water, 50 μL acid molybdate, and 12.5 μL Fisk&Subarow reducer to 50 μL of each sample, they were incubated at room temperature for 30 min. Finally, the absorbance at 700 nm was measured, the amount of phosphate (in μg) was determined according to a previously established standard curve, and the difference (Δμg phosphate) between blank and test related to CK activity units. All data were normalized relative to the total amount of proteins.

Isolation and preparation of cytosolic and mitochondrial fractions Experiments were performed according to the manufacturer's instructions (Merck KgaA, Darmstadt, Germany).

Statistical analysis Statistical evaluation of data was performed using a two-tailed Student's *t* test. Normal distribution was checked via the Shapiro–Wilks-W-Test.

Error bars indicate standard deviation (SD).

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References

Balsom PD, Soderlund K, Ekbolm B: Creatine in humans with special reference to creatine supplementation. Sports Med 18:268–280, 1994

Bessman SP, Carpenter CL: The creatine–creatine phosphate energy shuttle. Annu Rev Biochem 54:831–862, 1985

Bladier C, Wolvetang EJ, Hutchinson P, deHaan JB, Kola I: Response of a primary human fibroblasts cell line to H_2O_2: Senescence like growth arrest or apoptosis? Cell Growth Differ 8:589–598, 1997

Bogatskaia LN, Shegera VA: Creatine kinase activity and isoenzymic spectrum of myocardium creatine kinase in rats of different age. Ukr Biokhim 53: 71–74, 1981

Campisi J: The role of cellular senescence in skin aging. J Investig Dermatol Symp Proc 3:1–5, 1998

Carmeli E, Coleman R, Reznick AZ: The biochemistry of ageing muscle. Exp Gerontol 37:477–489, 2002

Chen Q, Ames BN: Senescence-like growth arrest induced by hydrogen peroxide in human diploid fibroblasts F65 cells. Proc Natl Acad Sci USA 91: 4130–4134, 1994

Chen Q, Bartholomew JC, Campisi J, Acosta M, Reagan JD, Ames BN: Molecular analysis of H_2O_2-induced senescent-like growth arrest in normal human fibroblasts: p53 and Rb control G1 arrest but not cell replication. Biochem J 332:43–50, 1998

Chung JH, Yano K, Lee MK, *et al*: Differential effects of photoageing vs intrinsic ageing on the vascularization of human skin. Arch Dermatol 138: 1437–1442, 2002

Cossarizza A, Baccarani-Contri M, Kalashnikova G, Franceschi C: A new method for the cytofluorimetric analysis of mitochondrial membrane potential using the J-aggregate forming lipophilic cation 5,5′,6,6′-tetrachloro-1,1′, 3,3′-tetraethylbenzimidazolcarbo-cyanine iodide (JC-1). Biochem Biophys Res Commun 197:40–45, 1993

Cotgreave IA, Weis M, Berggren M, Sandy MS, Moldeus PW: Determination of the intracellular protein thiol distribution of hepatocytes using monobromobimane derivatisation of intact cells and isolated subcellular fractions. J Biochem Biophys Methods 16:247–254, 1988

Crozatier B, Badoual T, Boehm E, *et al*: Role of creatine kinase in cardiac excitation–contraction coupling: Studies in creatine kinase-deficient mice. FASEB J 16:653–660, 2002

Daly MM, Seifter S: Uptake of creatine by cultured cells. Arch Biochem Biophys 203:317–324, 1980

Dodd JR, Christie DL: Cysteine 144 in the third transmembrane domain of the creatine transporter is located close to a substrate-binding site. J Biol Chem 276:46983–46988, 2001

Dolder M, Walzel B, Speer O, Schlattner U, Wallimann T: Inhibition of the mitochondrial permeability transition by creatine kinase substrates. Requirement for microcompartmentation. J Biol Chem 278:17760–17766, 2003

Dolder M, Wendt S, Wallimann T: Mitochondrial creatine kinase in contact sites: Interaction with porin and adenine nucleotide translocase, role in permeability transition and sensitivity to oxidative damage. Biol Signal Recept 10:93–111, 2001

Durand RE, Olive PL: Flow cytometry techniques for studying cellular thiols. Radiat Res 95:456–470, 1983

Ermini M: Ageing changes in mammalian skeletal muscle: Biochemical studies. Gerontology 22:301–316, 1976

Fisher GJ, Wang ZQ, Datta SC, Varani J, Kang S, Voorhees JJ: Pathophysiology of premature skin ageing induced by ultraviolet light. N Engl J Med 337:1419–1428, 1997

Gorselink M, Drost MR, Coumans WA, van Kranenburg GP, Hesselink RP, van der Vusse GJ: Impaired muscular contractile performance and adenine nucleotide handling in creatine kinase-deficient mice. Am J Physiol Endocrinol Metab 281:E619–E625, 2001

Hagen TM, Yowe DL, Bartholomew JC, Wehr CM, Do KL, Park JY, Ames BN: Mitochondrial decay in hepatocytes from old rats: Membrane potential declines, heterogeneity and oxidants increase. Proc Natl Acad Sci USA 94:3064–3069, 1997

Harman D: Ageing: A theory based on free radical and radiation chemistry. J Gerontol 11:298–300, 1956

Hausmann ON, Fouad K, Wallimann T, Schwab ME: Protective effects of oral creatine supplementation on spinal cord injury in rats. Spinal Cord 40: 449–456, 2002

Janssen E, Terzic A, Wieringa B, Dzeja PP: Impaired intracellular energetic communication in muscles from creatine kinase and adenylate kinase (M-CK/AK1) double knock-out mice. J Biol Chem 278:30441–30449, 2003

Kiistala U: Suction blister device for separation of viable epidermis from dermis. J Invest Dermatol 50:129–137, 1968

Krutmann J: New developments in photoprotection of human skin. Skin Pharmacol Appl Skin Physiol 14:401–407, 2001

Lawler JM, Barnes WS, Wu G, Song W, Demaree S: Direct antioxidant properties of creatine. Biochem Biophys Res Commun 290:47–52, 2002

Lee HC: Role of Mitochondria in human ageing. J Biomed Sci 4:319–326, 1997

Leuzzi V: Inborn errors of creatine metabolism and epilepsy: Clinical features, diagnosis, and treatment. J Child Neurol 17:3S89–3S97; discussion 3S97, 2002

McCully KK, Forciea MA, Hack LM, *et al*: Muscle metabolism in older subjects using 32P magnetic resonance spectroscopy. Can J Physiol Pharmacol 69:576–580, 1991

Niggli HJ, Applegate LA: Glutathione response after UVA irradiation in mitotic and postmitotic human skin fibroblasts and keratinocytes. Photochem Photobiol 65:680–684, 1997

Nohl H, Staniek K, Gille L: Imbalance of oxygen activation and energy metabolism as a consequence or mediator of ageing. Exp Gerontol 32: 485–500, 1997

Paavonen T, Aronen H, Saarelainen I, Neittaanmäki H, Hjelm I, Kiistala U: The BB-isozyme is a major component of creatine kinase in skin blister fluid. Brit J Dermatol 118:753–757, 1988

Pastoris O, Boschi F, Verri M, *et al*: The effects of ageing on enzyme activities and metabolite concentrations in skeletal muscle from sedentary male and female subjects. Exp Gerontol 35:95–104, 2000

Peral MJ, Garcia-Delgado M, Calonge ML, *et al*: Human, rat and chicken small intestinal Na^+–Cl^--creatine transporter: Functional, molecular characterization and localization. J Physiol 545 (Pt 1):133–144, 2002

Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C: Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Rad Biol Med 26:1231–1237, 1999

Ronquist G, Andersson A, Bendsoe N, Falck B: Human epidermal energy metabolism is functionally anaerobic. Exp Dermatol 12:572–579, 2003

Rosenkranz AR, Schmaldienst S, Stuhlmeier KM, Chen W, Knapp W, Zlabinger GJ: A microplate assay for the detection of oxidative products using 2′,7′-dichlorofluorescin-diacetate. J Immunol Methods 156:39–45, 1992

Rottenberg H, Wu S: Mitochondrial dysfunction in lymphocytes from old mice: Enhanced activation of the permeability transition. Biochem Biophys Res Commun 240:68–74, 1997

Scharffetter-Kochanek K, Wlaschek M, Brenneisen P, Schauen M, Blaudschun R, Wenk J: UV-induced reactive oxygen species in photocarcinogenesis and photoageing. Biol Chem 378:1247–1257, 1997

Schedel JM, Tanaka H, Kiyonaga A, Shindo M, Schutz Y: Acute creatine ingestion in human: Consequences on serum creatine and creatinine concentrations. Life Sci 65:2463–2470, 1999

Schlattner U, Mockli N, Speer O, Werner S, Wallimann T: Creatine kinase and creatine transporter in normal, wounded, and diseased skin. J Invest Dermatol 118:416–423, 2002

Sies H: Oxidative Stress. London: Academic Press, 1985

Snow RJ, Murphy RM: Creatine and the creatine transporter: A review. Mol Cell Biochem 224:169–181, 2001

Sohal RS: Hydrogen peroxide production by mitochondria may be a biomarker of ageing. Mech Ageing Dev 60:189–198, 1991

Speer O, Neukomm LJ, Murphy RM, *et al*: Creatine transporters: A reappraisal. Mol Cell Biochem 256/257:407–424, 2004

Stachowiak O, Dolder M, Wallimann T, Richter C: Mitochondrial creatine kinase is a prime target of peroxynitrite-induced modification and inactivation. J Biol Chem 273:16694–16699, 1998

Stead LM, Au KP, Jacobs RL, Brosnan ME, Brosnan JT: Methylation demand and homocysteine metabolism: Effects of dietary provision of creatine and guanidinoacetate. Am J Physiol Endocrinol Metab 281:E1095–E1100, 2001

Steeghs K, Benders A, Oerlemans F, *et al*: Altered Ca^{2+} responses in muscles with combined mitochondrial and cytosolic creatine kinase deficiencies. Cell 89:93–103, 1997

Steinhagen-Thiessen E, Hilz H: The age-dependent decrease in creatine kinase and aldolase activities in human striated muscle is not caused by an accumulationn of faulty proteins. Mech Ageing Dev 5:447–457, 1976

Sullivan PG, Geiger JD, Mattson MP, Scheff SW: Dietary supplement creatine protects against traumatic brain injury. Ann Neurol 48:723–729, 2000

Suzuki YJ, Forman HJ, Sevanian A: Oxidants as stimulators of signal transduction. Free Radic Biol Med 22:269–285, 1997

Vanakoski J, Kosunen V, Meririnne E, Seppala T: Creatine and caffeine in anaerobic and aerobic exercise: Effects on physical performance and pharmacokinetic considerations. Int J Clin Pharmacol Ther 36:258–262, 1998

Verzar F, Ermini M: Decrease of creatine-phosphate restitution of muscle in old age and the influence of glucose. Gerontologia 16:223–230, 1970

Walker JB: Creatine: Biosynthesis, regulation, and function. Adv Enzymol 50: 177–252, 1979

Wallimann T, Dolder M, Schlattner U, *et al*: Some new aspects of creatine kinase (CK): Compartmentation, structure, function and regulation for cellular

and mitochondrial bioenergetics and physiology. BioFactors 8:229–234, 1998

Wallimann T, Wyss M, Brdiczka D, Nicolay K, Eppenberger HM: Intracellular compartmentation, structure and function of creatine kinase isoenzymes in tissues with high and fluctuating energy demands: The "phosphocreatine circuit" for cellular energy homeostasis. Biochem J 281:21–40, 1992

Wendt S, Schlattner U, Wallimann T: Differential effects of peroxynitrite on human mitochondrial creatine kinase isoenzymes. Inactivation, octamer destabilization, and identification of involved residues. J Biol Chem 278: 1125–1130, 2003

Wyss M, Schulze A: Health implications of creatine: Can oral creatine supplementation protect against neurological and atherosclerotic disease? Neuroscience 112:243–260, 2002