

Antioxidants prevent health-promoting effects of physical exercise in humans

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Exercise promotes longevity and ameliorates type 2 diabetes mellitus and insulin resistance. However, exercise also increases mitochondrial formation of presumably harmful reactive oxygen species (ROS). Antioxidants are widely used as supplements but whether they affect the health-promoting effects of exercise is unknown. We evaluated the effects of a combination of vitamin C (1000 mg/day) and vitamin E (400 IU/day) on insulin sensitivity as measured by glucose infusion rates (GIR) during a hyperinsulinemic, euglycemic clamp in previously untrained ($n = 19$) and pre-trained ($n = 20$) healthy young men. Before and after a 4 week intervention of physical exercise, GIR was determined, and muscle biopsies for gene expression analyses as well as plasma samples were obtained to compare changes over baseline and potential influences of vitamins on exercise effects. Exercise increased parameters of insulin sensitivity (GIR and plasma adiponectin) only in the absence of antioxidants in both previously untrained ($P < 0.001$) and pre-trained ($P < 0.001$) individuals. This was paralleled by increased expression of ROS-sensitive transcriptional regulators of insulin sensitivity and ROS defense capacity, peroxisome-proliferator-activated receptor gamma (PPAR γ), and PPAR γ coactivators PGC1 α and PGC1 β only in the absence of antioxidants ($P < 0.001$ for all). Molecular mediators of endogenous ROS defense (superoxide dismutases 1 and 2; glutathione peroxidase) were also induced by exercise, and this effect too was blocked by antioxidant supplementation. Consistent with the concept of mitohormesis, exercise-induced oxidative stress ameliorates insulin resistance and causes an adaptive response promoting endogenous antioxidant defense capacity. Supplementation with antioxidants may preclude these health-promoting effects of exercise in humans.

aging | hormesis | insulin resistance | oxidative stress | reactive oxygen species

Type 2 diabetes mellitus is increasing worldwide at epidemic rates and is associated with both microvascular and macrovascular complications (1). Type 2 diabetes mellitus is caused by a combination of insulin resistance involving a number of peripheral tissues, including skeletal muscle (2, 3), and an inadequate β -cell response despite normal or even increased amounts of circulating insulin.

Physical exercise exerts numerous favorable effects on general health (4) and specifically has been shown to improve glucose metabolism in the insulin-resistant state (5). This effect may be independent of exercise-related changes in body mass (6). Moreover, physical exercise has been shown to be effective in preventing type 2 diabetes in high risk individuals (7, 8) and may be even more effective than the most widely used anti-diabetic drug, metformin (9).

These beneficial effects of physical exercise on insulin resistance involve multiple mechanisms, including enhanced expression of glucose transporters and translocation of glucose transporters to the plasma membrane independent of insulin (10). Exercise, as well as weight loss, has been linked to activation of mitochondrial metabolism, and reduced mitochondrial metab-

olism has been functionally connected with type 2 diabetes (11). Mitochondria, however, are also the main source of reactive oxygen species (ROS), which are inevitable by-products of oxidative glucose metabolism. Muscle is also known to generate free radicals, especially during contraction and physical exercise (12). It has been suggested that ROS may mediate some health-promoting effects, at least in nonprimate model systems (13–17).

We here evaluated the possibility that ROS are required for the insulin-sensitizing capabilities of physical exercise in healthy humans and that commonly used antioxidants, such as vitamin C and vitamin E, may abrogate the health-promoting effects of both physical exercise and oxidative stress in humans.

Results

Baseline Characteristics. Of the 40 individuals included in the present study, 20 were known to be previously trained, and 20 were previously untrained. Study subject characteristics in the preinterventional state are given in Table 1. No significant differences in age, height, body mass index, fat free mass, or VO₂ maximum were observed within the groups (Table 1) and no significant differences in age, height and body mass index were observed between untrained and pre-trained groups. Not surprisingly, pre-trained individuals had a significantly higher fat free mass ($P = 0.03$) and VO₂ maximum ($P < 0.001$).

Half of the previously untrained and previously trained groups were randomly assigned to either antioxidant supplementation as described in *Methods* or to no supplementation (creating 4 groups of 10 each) (supporting information (SI) Fig. S1). All subjects underwent a 4 week exercise training program irrespective of antioxidant supplementation and previous training status. One untrained individual withdrew during the study for personal reasons unrelated to the experimental protocol.

Induction of Oxidative Stress by Short-Term Exercise. It is well-established that physical exercise increases ROS formation in skeletal muscle (12); however, it is not known if the health-promoting effects of exercise are partly due to this effect. To replicate the ROS-inducing capacity of exercise in our specific experimental set-up, we subjected previously untrained individuals to 3 days of exercise with muscle biopsy before (Fig. S1, “pre”) and after this short-term intervention (Fig. S1, “early”). We measured concentrations of thiobarbituric acid-reactive

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Table 1. Baseline characteristics of study subjects

	Previously untrained study group					Pretrained study group				
	No supplements		Vitamin C/Vitamin E		P-Value	No supplements		Vitamin C/Vitamin E		P-Value
	Mean	SD	Mean	SD		Mean	SD	Mean	SD	
Number/sex	10/male	n.a.	10/male	n.a.	n.a.	10/male	n.a.	10/male	n.a.	n.a.
Age, years	26.70	4.34	27.44	3.02	0.69	25.40	2.15	26.00	1.95	0.54
Height, cm	179.20	4.98	182.32	8.83	0.38	182.96	3.87	177.91	7.16	0.08
Body Mass Index, kg/m ²	24.37	2.53	24.19	1.84	0.87	24.33	1.31	23.33	1.36	0.13
Fat free mass, kg	60.64	5.88	66.20	7.28	0.10	69.71	3.85	65.84	5.31	0.09
VO ₂ max, ml/min·kg ⁻¹	45.85	5.46	45.21	7.03	0.84	54.42	4.90	54.31	5.10	0.96

n.a. = not applicable.

substances (TBARS), a well-established marker of overall oxidative stress reflecting oxidized lipids and thus ROS formation in mammals, within skeletal muscle of these previously untrained individuals in the presence or absence of antioxidant treatment. As expected (12), we observed a more than 2-fold increase in oxidative stress, as reflected by TBARS levels, following physical exercise in the absence of antioxidants (Fig. S2, Left pair of bars, $P = 0.008$). By contrast, those individuals taking antioxidant supplements showed no significant increase in muscle TBARS levels after exercise (Fig. S2, Right pair of bars, $P = 0.19$) resulting in significantly reduced TBARS formation after 3 days of exercise in comparison to untreated individuals (Fig. S2, shaded bars, $P = 0.03$). Thus, consistent with previous findings (12), these observations suggest that short-term physical exercise induces skeletal muscle ROS formation and that antioxidant supplements reduce this formation, at least during the first 3 days.

Antioxidants Prevent Increase of Insulin Sensitivity Following Physical Exercise.

Glucose infusion rates. Physical training has been shown to ameliorate insulin resistance and to improve glucose metabolism (5). Thus, both previously untrained and previously trained individuals were subjected to training with twenty 85 min sessions of defined physical exercise on 5 days per week (with or without addition of antioxidant supplementation) with measurement of insulin sensitivity by glucose infusion rates (GIR) during a hyperinsulinemic euglycemic clamp (18). As expected (5), nonsupplemented individuals showed a significant increase in GIR, i.e., increased insulin sensitivity after 4 weeks of exercise irrespective of previous training status (previously untrained: Fig. 1A, Left pair of bars, $P < 0.001$; and previously trained: Fig. 1B, Left pair of bars, $P < 0.001$), confirming previous findings that physical exercise induces an increase in insulin sensitivity. By contrast, neither previously untrained individuals and pretrained individuals who received antioxidants exhibited significant changes in GIR following exercise (Fig. 1A, Right pair of bars, $P = 0.07$, and B, Right pair of bars, $P = 0.89$). Thus physical exercise induced an increase in insulin sensitivity only in the absence of antioxidants. The same impaired effect of exercise on GIRs seen in the antioxidant-treated group was also apparent in the previously untrained individuals when the supplemented individuals were compared to nonsupplemented individuals (Fig. 1A, shaded bars, $P = 0.003$). Finally, analysis of all 39 individuals, irrespective of straining status, demonstrates a highly significant (Fig. S3A, $P < 0.001$ for ANOVA) effect of antioxidant supplementation on the blockage of exercise-induced improvement of GIR. Interestingly, inclusion of the baseline training status in the statistical analyses revealed that the effect of exercise on GIR was independent of the pretraining status of the study participants ($P = 0.58$ for interaction term of pretraining status by ANOVA), indicating that antioxidants can abolish the insulin sensitizing effects of exercise in both untrained and previously well-trained subjects. We additionally

measured serum concentrations of TBARS. When comparing these to GIRs in the postinterventional state in all 39 individuals, we observed a significant correlation with TBARS serum levels (Pearson's $r = 0.353$, $P < 0.05$); this suggests that TBARS serum levels, at least to some extent, correlate with insulin sensitivity irrespective of antioxidant supplementation and previous training status, whereas no significant effect of antioxidant supplementation on postinterventional TBARS levels was observed ($P = 0.73$ for ANOVA, and $P = 0.43$ for interaction of vitamins by pretraining status by ANOVA).

Additional plasma markers of insulin sensitivity. Plasma concentrations of the adipocyte-derived secretory protein adiponectin have been shown to be positively correlated with insulin sensitivity in humans and inversely correlated with type 2 diabetes risk (19). We observe an increase in circulating adiponectin levels following physical exercise in both previously untrained individuals (Fig. 1C, Left pair of bars, $P < 0.001$) and pretrained individuals (Fig. 1D, Left pair of bars, $P < 0.001$). In contrast, previously

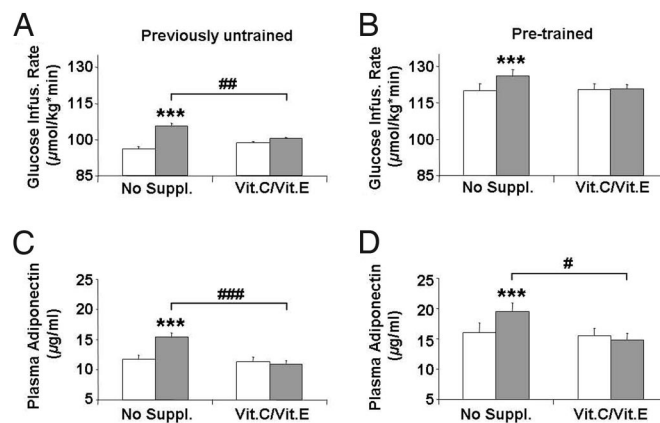


Fig. 1. Antioxidants prevent exercise-dependent induction of insulin sensitivity. (A) Glucose infusion rates (GIR) during euglycemic hyperinsulinemic clamps in previously untrained individuals before (white bars) and after (shaded bars) physical exercise over 4 weeks. (Left pair of bars) Individuals not taking any medication or placebo; (Right pair of bars) individuals taking both vitamin C (1000 mg/day) as well as vitamin E (400 IU/day). Bars depict means, error bars show standard error means (applies to all subsequent panels and figures). Significances (applies to all subsequent panels and Fig. 3): * indicates $0.01 < P < 0.05$ comparing data before and after 4 weeks of exercise, # indicates $0.01 < P < 0.05$ comparing "no suppl." with "Vit.C/Vit.E" groups after intervention, ** indicates $0.001 \leq P \leq 0.01$ comparing data before and after 4 weeks of exercise, ## indicates $0.001 \leq P \leq 0.01$ comparing "no suppl." with "Vit.C/Vit.E" groups after intervention, *** indicates $P < 0.001$ comparing data before and after 4 weeks of exercise, ### indicates $P < 0.001$ comparing "no suppl." with "Vit.C/Vit.E" groups after intervention. (B) The same set of data derived from a physically pretrained group of individuals. (C) Plasma adiponectin levels in the previously untrained and (D) pretrained individuals. (D) state.

intervention and antioxidant supplementation on expression of these genes ($P = 0.21$ to 0.89 for interaction of vitamins by pretraining-status by ANOVA).

Taken together, these findings indicate that physical exercise induces several molecular regulators of insulin sensitivity irrespective of previous training status and that this induction is widely inhibited by antioxidant supplementation.

Molecular Promotion of Muscle Antioxidant Defense Following Physical Exercise Is Abrogated by Antioxidants. The transcriptional coactivators PGC1 α and PGC1 β have not only been linked to increased insulin sensitivity but have also been shown to induce expression of several enzymes known to be involved into detoxification of reactive oxygen species (ROS), including superoxide dismutase 2 (SOD2), glutathione peroxidase 1 (GPx1) and possibly other enzymes of similar biochemical function (20). Accordingly, in the present study, physical exercise resulted in a strongly increased expression of *SOD1* (Fig. 2 G and H, Left pair of bars, $P < 0.05$ to $P < 0.001$), *SOD2* (Fig. 2 I and J, Left pair of bars, $P < 0.05$ to $P < 0.001$), and *GPx1* (Fig. 2 K and L, Left pair of bars, $P < 0.001$) in previously untrained and previously trained, antioxidant naive individuals, whereas pretreatment with antioxidants prevented this induction (Fig. 2 G, I, and K, Right pair of bars, $P = 0.92$, $P = 0.06$, and $P = 0.10$, respectively). Similar while less pronounced effects were observed for catalase (*CAT*) ($P = 0.045$ and $P = 0.13$, not depicted). When comparing the relative expression of these enzyme-encoding mRNAs in the postinterventional state in the presence and absence of antioxidants, exercise increased expression to a much lesser, if any, extent in antioxidant-supplemented individuals whether previously untrained or previously trained (Fig. 2 G-L, shaded bars, $P < 0.05$ to $P < 0.001$). As observed for PPAR γ , PGC1 α , and PGC1 β , we found a strong effect of antioxidant supplementation to block exercise training-induced expression of antioxidant enzyme mRNAs for the entire sample of previously trained and untrained individuals ($n = 39$) (Fig. S4 D-F) (*SOD1*: $P < 0.001$; *SOD2*: $P = 0.01$; *GPx1*: $P < 0.001$; and *CAT*: $P = 0.81$, all for ANOVA). In some of these cases, previous training status had an impact on the effects of exercise intervention and antioxidant supplementation on 2 of the expression levels (*SOD1*: $P = 0.003$; *SOD2*: $P = 0.32$; *GPx1*: $P = 0.046$; and *CAT*: $P = 0.83$, all for interaction of vitamins by pretraining-status by ANOVA). However, this effect was restricted to *SOD1* and *GPx1*, while, notably, the mitochondrially active *SOD2* appeared to be unaffected by previous training status.

Taken together, physical exercise induces numerous molecular regulators of insulin sensitivity and antioxidant defense, most of which are almost completely inhibited by antioxidant pretreatment in healthy young men (Fig. 3).

Discussion

Based on the evidence derived from the current study, we here propose an essential role for exercise-induced ROS formation in promoting insulin sensitivity in humans. This induction appears to involve the ROS-dependent transcriptional coactivators PGC1 α and PGC1 β , and the transcription factor PPAR γ and their targets *SOD1*, *SOD2*, *GPx1*, and, to a reduced extent, *CAT*. Most importantly, these changes in gene expression and the increase in insulin sensitivity following physical exercise are almost completely abrogated by daily ingestion of the commonly used antioxidants vitamin C and vitamin E. Thus, antioxidant supplementation blocks many of the beneficial effects of exercise on metabolism.

This direct molecular link between exercise-dependent formation of ROS, activation of PGC1 α , PGC1 β and PPAR γ on the one hand and increased insulin sensitivity on the other hand, strongly suggest that oxidative stress can be instrumental in preventing type 2-diabetes. The transcriptional coactivator

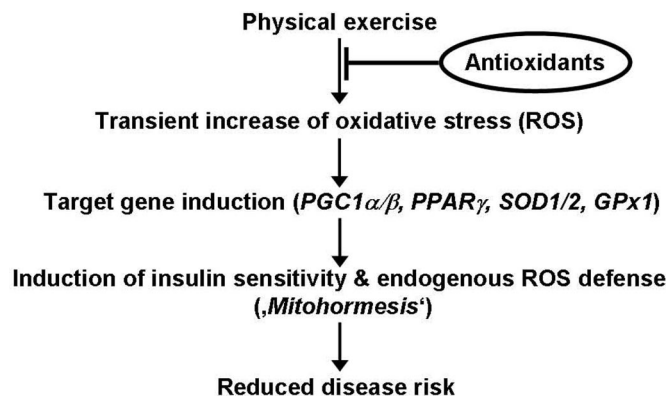


Fig. 3. Mitohormesis links physical exercise and subsequent formation of reactive oxygen species to insulin sensitivity and antioxidant defense. Physical exercise exerts ameliorating effects on insulin resistance by increasing mitochondrial formation of reactive oxygen species in skeletal muscle to induce expression of PGC1 α , PGC1 β , and PPAR γ as inducers of insulin sensitivity, as well as superoxide dismutases 1 and 2 and glutathione peroxidase 1, key enzymes of ROS defense. Notably, by blocking exercise-dependent formation of reactive oxygen species due to ingestion of antioxidant supplements, health promoting effects of physical exercise are abolished, and physical exercise fails to promote insulin sensitivity and antioxidant defense in the presence of vitamin C and vitamin E.

PGC1 α has been previously linked to type 2 diabetes in humans (21, 22). This protein has also been shown to be inducible by various oxidative stressors (20), as well as physical exercise in rodents, notably in a vitamin C sensitive manner (17). Given its synergistic potency in coactivating the transcription factor PPAR γ to promote insulin sensitivity, the previously established role of PGC1 α as a ROS sensor in neurons that in turn induces ROS defense (20), as well as in rodent muscle (17), suggests that activation of PGC1 α and possibly PGC1 β may be important factors in promoting insulin sensitivity by both exercise and ROS in skeletal muscle. Moreover, in addition to the increase in insulin sensitivity following exercise-induced ROS formation, we also observe an induction of all relevant ROS defense enzyme expression levels, namely *SOD1* and *SOD2*, *GPx1*, and, to a reduced extent, *CAT*. Notably, some of these enzymes have been previously linked to transcriptional promotion by PGC1 α (20), and, at least for *SOD2*, exercise has been shown to induce its expression in rodents (23).

Nevertheless, the published evidence is ambiguous with a number of studies suggesting that exposure to ROS may promote insulin resistance (24, 25) whereas others find the opposite (14). In the present study, we find that increased ROS formation efficiently counteracts insulin resistance. Previously published findings in nonprimate models also support this interpretation (13–17). One possible explanation for the apparent conflict between the different studies may be that those studies suggesting an inverse relation between ROS and insulin sensitivity were obtained in models of continuous exposure to increased levels of ROS (24, 25), whereas our current findings and those of other studies (23, 26) may reflect transient increases in ROS during limited periods of physical exercise only.

This notion is further supported by the fact that most negative effects of antioxidant supplements observed in the current study occur irrespective of previous training status. While most effects appear quantitatively more pronounced within the previously untrained study group (i.e., Left arms of Figs. 1, 2, and S1), the data do not support the assumption that antioxidant supplement intake is less detrimental in previously trained subjects. ANOVA, which considered covariables as stated in Methods, indicates that most effects of antioxidants are similar in both pre-trained and untrained individuals (Fig. S3 and Fig. S4) and

according to the manufacturer's instructions. Samples were incubated in the ABI PRISM 7000 sequence detector for an initial denaturation at 95 °C for 10 min, followed by 40 PCR cycles, each cycle consisting of 95 °C for 15 s, 60 °C for 1 min, and 72 °C for 1 min. The following primers were used: human *PGC-1 α* : 5'TGCCTGGATTGTTGACATGA (sense) and 5'TTTGTCAGGCTGGGGTAGG (antisense); human *PGC-1 β* : 5'TTGAGGAGTGCAGGTGCTG (sense) and 5'ATCTGGGCGACGAGAAGTGC (antisense), human *PPAR γ* : 5'AGGCGAGGCGATCTTGACAG (sense) and 5'GATGCGGATGGCCACCTCTTT (antisense); human superoxide dismutase 1 (*SOD1*): 5'GGTGTGGCCGATGTGCTATT (sense) and 5'CTGCTTTTTCATGCACCACA (antisense); human *SOD2*: 5'TGCTGCTTGTCCAAATCAGG (sense) and 5'CACACATCAATCCCCAGCAGT (antisense); human *Gpx1*: 5'GCGGCGCCAGTCGGTGA (sense) and 5'GAGCTTGGGGTCG-GTCATAA (antisense); human catalase: 5'TCCGGGATCTTTTAAACGCCATTG (sense) and 5'TCGAGCAGGTAGGGACAGTTTAC (antisense); human *18S* rRNA: 5'TGCCATGTCTAAGTACGCACG (sense); 5'TTGATAGGGCAGACGTTTCA (antisense). SYBR Green I fluorescence emissions were monitored after each cycle. Expression of *PGC-1 α* , *PGC-1 β* , *PPAR γ* , *SOD1*, *SOD2*, *Gpx1*, *CAT*, and *18S* rRNA were quantified by using the second derivative maximum method of the TaqMan Software (Applied Biosystems) determining the crossing points of individual samples by an algorithm which identifies the first turning point of the fluorescence curve. Amplification of specific transcripts was confirmed by melting curve profiles (cooling the sample to 68 °C and reheating slowly up to 95 °C with parallel measurements of fluorescence) at the end of each PCR. The specificity of the PCR was further verified by subjecting the amplification products to agarose gel electrophoresis.

Statistical Analysis. All data collection processes and subsequent statistical analyses were performed with SPSS, Version 15.0. Variables were tested for normal distribution using the Kolmogorov-Smirnov test. Nonnormally distributed variables were log transformed to approximate a normal distribution

before applying *t* test or general linear modeling statistics. P-values of less than 0.05 were considered significantly different.

Group comparisons (Table 1) were made using a 2-sided unpaired Student's *t* tests. Within previously untrained ($n = 20$) or pretrained ($n = 20$) subgroups, interindividual effects of antioxidant treatment between treatment groups at baseline, as well as after intervention, were compared with two-sided unpaired Student's *t* tests. For comparing intra-individual effects of exercise within treatment groups (pre- vs. postexercise), 2-sided paired Student's *t* tests were used (Table 1 and Figs. 1 and 2).

To determine putatively differential effects of initial training status (previously untrained vs. pretrained), multivariate analyses were performed using a general linear model approach on the delta values (postvalues minus prevalues) of all 39 individuals. The ANOVA statistics for effects of vitamin supplements as dependent variable are given after adjustment for the covariates "open" versus "blinded", and "initial training status" (previously untrained vs. pretrained). A significant P value would indicate that vitamin supplementation has an effect on outcome measurements irrespective of initial training status. This specific test has been denominated "ANOVA" throughout the *Results* and *Discussion* sections.

We also performed an additional ANOVA including an interaction term (training status by supplement) again using delta values (postvalues minus prevalues) and adjusting for the above covariates. A significant P value would indicate that the effect of vitamin supplementation is dependent on initial training status. This test has been denominated "interaction of vitamins by pretraining-status by ANOVA" throughout the *Results* and *Discussion* sections.

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