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ORIGINAL ARTICLE

Katja Heinemeier · Henning Langberg · Michael Kjaer

Exercise-induced changes in circulating levels of transforming growth factor-β-1 in humans: methodological considerations

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Abstract Mechanical loading of cells induces the expression of transforming growth factor-β-1, and acute exercise, which involves mechanical loading of several tissues, could thus increase its circulating level in humans. However, no consensus exists regarding the plasma concentration of this cytokine in resting subjects (reported values range from 500 to 18,300 pg ml^{-1}) and also the extent of intra-individual variation is unknown. As a basis for detecting exercise-induced changes in transforming growth factor-β-1, we measured its concentration, by enzyme-linked immunosorbent assay, in plasma from eight healthy resting subjects. Plasma was sampled from each subject on five successive days according to a procedure designed to minimize activation of platelets, as platelet α-granules contain large amounts of transforming growth factor-β-1. The mean plasma level was relatively low [1155 (30) pg ml^{-1}, mean (SE)], and did not differ between days, indicating that platelet activation was minimal. Several alterations in the blood sampling procedure did not affect results, while a 40% increase was seen when blood was not cooled appropriately prior to centrifugation. A moderate intra-individual variation (average CV = 9.8%) indicated a stable plasma level at rest. In response to exercise (1 h of treadmill running) the plasma concentration of transforming growth factor-β-1 increased from 992 (49) pg ml^{-1} (at rest) to 1301 (39) pg ml^{-1} (post exercise) ($P < 0.05$) ($n = 6$). In conclusion, the resting plasma level of transforming growth factor-β-1 was stable over time when blood samples were treated appropriately. Exercise increased the plasma concentration, perhaps indicating a release from mechanically loaded tissues.

Keywords Exercise · Plasma · Platelets · TGF-β-1

K. Heinemeier (✉) · H. Langberg · M. Kjaer
Sports Medicine Research Unit, Bispebjerg Hospital, Building 8, 1st floor, 23 Bispebjerg Bakke, 2400 Copenhagen NV, Denmark
E-mail: kh08@bbh.hosp.dk
Tel.: +45-353-16089
Fax: +45-353-12733

Introduction

Transforming growth factor β (TGF-β) is a multifunctional 25-kDa dimeric polypeptide expressed as three different isoforms in mammals (TGF-β-1 to -3) (Gleizes et al. 1997). The TGF-β-1 isoform is involved in regulation of cell proliferation, immune function, and extra cellular matrix (ECM) production. It induces a net ECM accumulation by stimulating the expression of ECM components (collagen, proteoglycans and fibronectin) (Ignotz and Massague 1986; Robbins et al. 1997) and by inhibiting the expression of ECM-degrading enzymes (Li et al. 2000). In connection with its stimulatory effect on collagen synthesis under normal physiological conditions, TGF-β-1 has also been shown to be involved in various fibrotic diseases, such as renal and cardiac fibrosis (Bitzer et al. 1998; Lijnen et al. 2000). Additionally, several studies indicate that the expression of TGF-β-1 is induced by mechanical loading of cells (Cillo et al. 2000; Gutierrez and Perr 1999; O'Callaghan and Williams 2000; Ruwhof et al. 2000; Skutek et al. 2001), and in relation to this TGF-β-1 is thought to play an important role in the increased type I collagen synthesis seen in response to mechanical loading of various tissues, such as bone and cardiac tissue (Villarreal and Dillmann 1992). Based on the evidence of mechanical regulation of TGF-β-1 expression, we hypothesized that the mechanical loading of tissue during exercise could lead to changes in the concentration of TGF-β-1 in plasma. However, measuring the content of TGF-β-1 in plasma has not previously been without problems, presumably explaining the lack of consensus in values of plasma-TGF-β-1 reported over recent years. Circulating levels in the resting state have been reported to range between 500 pg ml^{-1} (mean, $n = 10$) (Hering et al. 2002) and 18,300 pg ml^{-1} (mean, $n = 13$) (Flisiak et al. 2000) in healthy subjects. Moreover, no reports exist with regard to the extent of intra-individual variation in the TGF-β-1 plasma level, which could have implications for detecting changes in circulating TGF-β-1 in longitudinal

intervention studies, for example, in which individuals are subjected to physiological perturbations, such as acute exercise and physical training.

The variable results for plasma-TGF-β-1 in healthy subjects is presumably caused, at least in part, by the high concentration of TGF-β-1 in platelet α-granules (Assoian and Sporn 1986). In serum, obtained from blood where platelet activation is unrestricted, levels of TGF-β-1 have been found to be $\sim$20 times higher than in plasma, where platelet activation is minimized (Wakefield et al. 1995). The release of TGF-β-1 from platelet α-granules complicates the process of obtaining correct estimates of the physiological plasma-TGF-β concentration, as any in vivo or ex vivo platelet activation, during sampling and handling of blood, will lead to a considerable increase in the apparent plasma TGF-β-1 level (Grainger et al. 2000). The aim of the present study was to find a reliable method for sampling and handling of blood for measurement of TGF-β-1, and to investigate the extent of variation in individual levels of plasma-TGF-β-1. Bearing this in mind, we determined levels of plasma-TGF-β-1 both before and after an acute exercise bout.

Methods

Experiment 1 – rest

Plasma samples were obtained from eight healthy subjects (five females and three males) ranging in age from 24 to 32 years. All subjects gave informed consent, and the study was approved by the Ethical Committee of Copenhagen [(KF) 11–088/01]. The subjects fasted from 10 p.m. until blood was drawn the next day between 8.30 and 9.00 a.m. This procedure was repeated on five subsequent days. Blood was obtained and handled according to a protocol designed especially to minimize both in vivo and in vitro platelet activation, based on recommendations published earlier (Wakefield et al. 1995) and as given by the manufacturer of the ELISA kit used to measure TGF-β-1 (R&D systems, Minneapolis, USA). In order to minimize in vivo platelet degranulation, a butterfly-needle [Safety-lok 0.8×19 mm (21 G), Becton Dickinson] was used, as this should induce less trauma than a traditional needle (Wakefield et al. 1995). In addition, the tourniquet was always removed immediately after needle insertion in order to ensure as short a period of cuffing as possible. The tube containing the first 3 ml of blood was discarded as this is thought to be more degranulated than the following (Wakefield et al. 1995). Blood was sampled in tubes containing CTAD (citric acid, theophylline, adenosine, dipyridamole) – a cocktail that should be an especially effective inhibitor of ex-vivo platelet activation[1].

The collected samples were put on slush-ice immediately and left for 15–30 min before centrifugation at 1000 *g* at 4°C (this procedure was based on preliminary experiments showing lower TGF-β-1 levels in pre-cooled samples than in samples centrifuged immediately, which could be explained by a more rapid cooling of the blood in slush-ice than in a 4°C centrifuge and consequently a lesser degree of platelet activation in the pre-cooled samples). The top two-thirds of the resulting supernatant was at once carefully removed and centrifuged at 10,000 *g* at 4°C for 10 min (as recommended by R&D Systems). The supernatant obtained after the second centrifugation was aliquoted right away and stored at −80°C. To evaluate the importance of the precautions taken in order to minimize ex-vivo platelet activation, extra blood samples were obtained from each subject and handled differently with regards to cooling and sampling tubes. Extra samples, obtained on day 4, were not put on ice prior to the first centrifugation and on day 5 one extra set of samples was cooled for 90 min (instead of the maximum 30 min) and an additional set was sampled in K_3EDTA tubes (instead of CTAD). Otherwise the extra samples were treated exactly as described above.

Experiment II – exercise

Six healthy moderately trained male volunteers with a mean age of 26 years (range, 25–28 years) and a mean body mass of 77 kg (range, 70–80 kg) were included in the study. All subjects gave informed consent, and the study was approved by the Ethical Committee of Copenhagen [(KF) 11–088/01]. The participants performed 1 h of 3% uphill treadmill running, preceded by a warm-up of 10 min treadmill running at 10 km/h. In the present study no direct determination of the individual's maximal oxygen uptake ($\dot{V}O_{2max}$) was made, but all individuals were moderately trained and young, and they were all completely exhausted after 1 h, which indicates that the relative work intensity was between 70 and 80% of $\dot{V}O_{2max}$. Blood samples were obtained before exercise, immediately after and 6, 20 and 68 h post exercise. Subjects fasted from 10 p.m. on the day before the exercise intervention and on days preceding blood sampling. In addition they were told not to engage in any type of exercise/training for 48 h prior to the first day of the study and for the entire length of the experiment. Blood was drawn as described above and sampled in both CTAD tubes and K_3EDTA tubes. The blood samples obtained in CTAD tubes were handled as described above, while K_3EDTA samples were treated according to standard procedures for plasma samples obtained in our laboratory. This entailed 30 min of cooling on ice immediately after sampling and subsequent centrifugation at 3000 *g* for 15 min. Practically all supernatant (plasma) was transferred to Eppendorf tubes and frozen at −80°C until analysis.

Measurement of TGF-β-1

We decided to analyse samples for TGF-β-1 with a commercially available ELISA kit (DuoSet, R&D Systems, Minneapolis, USA), as it has been shown that the activation/dilution procedure (see below) employed in this assay does not lead to dilution non-linearity as opposed to several other commercial ELISA kits (Kropf et al. 1997). Prior to analysis all samples were activated (in order to separate TGF-β-1 from its binding proteins) by acidification, using 2.5 M acetic acid/10 M urea with an incubation time of 10 min. The activated samples were neutralized using 2.7 M NaOH/1 M HEPES and then diluted four times in 1.4% delipidized bovine serum (DuoSet diluent concentrate 1, R&D Systems), 0.05% Tween 20 in PBS, pH 7.2–7.4. Immediately after dilution the samples were loaded in duplicate on the ELISA plate. The average coefficient of variation (CV) on duplicate measurements was found to be 2.0% (range, 0.02–10.6%). The intra-assay precision was found to be 5.9% at 1619 pg ml^{-1} (plasma), while the inter-assay precision was 7.6% at 1724 pg ml^{-1} (plasma). Control samples containing known levels of TGF-β-1 were measured in each ELISA.

Correction for changes in plasma volume

In the exercise study changes in plasma volume were estimated by measuring levels of haematocrit by the micro-haematocrit method, and levels of TGF-β-1 were corrected for changes in plasma volume, relative to the resting value, according to the van Beaumont formula (van Beaumont et al. 1973).

[1] Theophylline, adenosine and dipyridamole increase the cAMP concentration in platelets, thereby inhibiting platelet activation. Citrate chelates calcium ions, which are necessary for the coagulation reaction (Mody et al. 1999).

Statistics

All data are presented as mean (SE) or range. Non-parametric tests were used for testing of significance, as the number of observations was relatively low and did not allow for determination of standard distribution. The Friedman test was used to investigate if significant differences were seen over time. If this was the case, a Wilcoxon signed ranks test was used to compare paired observations. Spearman rank test was used for correlation measurements. $P < 0.05$ was considered significant. CV was calculated as a measure of variation.

Results

A TGF-β-1 concentration of 1150 (30) pg ml^{-1} was found as a mean of all observed values in eight subjects on five successive days, and no significant difference was found between days ($P > 0.05$) (Fig. 1).

Significant differences were found between individuals [range 883 (68) pg ml^{-1} to 1485 (38) pg ml^{-1}] ($P < 0.001$) (Table 1) and the CV on individual plasma TGF-β-1 levels averaged 9.8% (range 4.9% to 17.1%) (Table 1).

When blood samples were not cooled on ice prior to the first centrifugation, levels of TGF-β-1 were 40% higher [1702 (162) pg ml^{-1}] than in controls [1212 (43) pg ml^{-1}] ($P = 0.01$) (Fig. 2), and the concentration of TGF-β-1 found in the non-cooled samples did not correlate with the concentrations found in controls ($r = 0.50$, $P > 0.05$) (Fig. 3a).

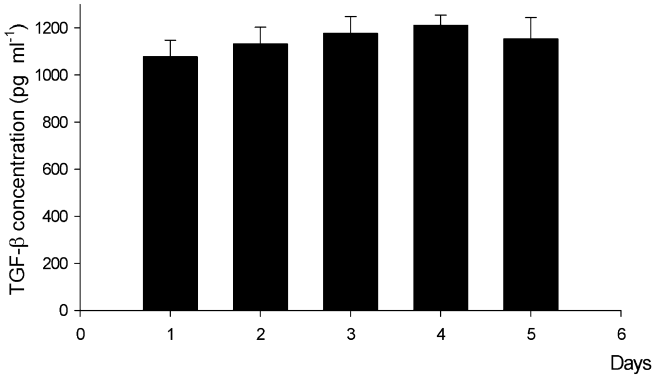

Fig. 1 The concentration of transforming growth factor-β (*TGF-β*) was measured in plasma obtained from eight healthy subjects on five successive days. Blood was sampled and handled according to a protocol designed to minimize in vivo and ex vivo platelet activation

Table 1 Levels of plasma-TGF-β in 8 healthy subjects

Subject no.	Plasma-TGF-β level (pg ml^{-1})	CV (%)
1	1109 (24)	4.9
2	1246 (52)	9.4
3	1035 (73)	15.7
4	1485 (38)	5.7
5	883 (68)	17.1
6	1111 (64)	12.9
7	1201 (43)	8.0
8	1133 (25)	4.9

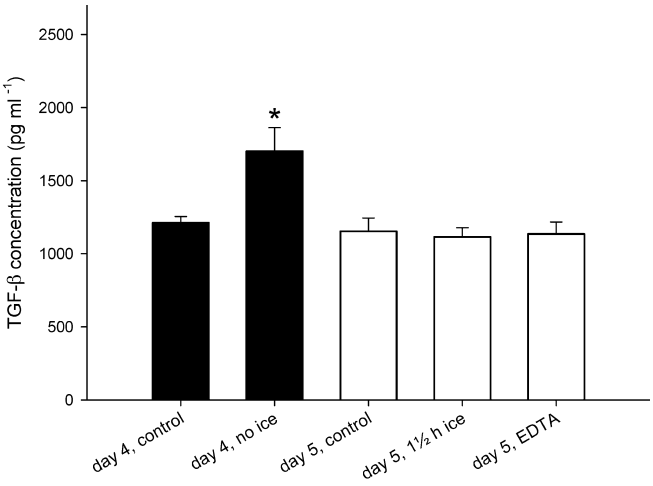

Fig. 2 TGF-β was measured in plasma obtained from extra blood samples drawn on day 4 and 5, which were either: (1) not put on ice prior the initial centrifugation ("*day 4, no ice*"), (2) left on ice for 90 min instead of 30 min maximum before the initial centrifugation ("*day 5, 1 1/2 h ice*") or (3) sampled in EDTA tubes instead of CTAD tubes ("*day 5, EDTA*") ($n = 8$). $^{*}P = 0.01$

No difference was seen between control samples and samples left on ice for 90 min (instead of max. 30 min) ($P > 0.05$) (Fig. 2) and a significant correlation was found in the level of TGF-β-1 in these two sets of samples ($r = 0.91$) ($P < 0.01$) (Fig. 3b). Likewise the concentration of TGF-β-1 in samples obtained in K_3EDTA tubes did not differ from control samples ($P > 0.05$) (Fig. 2) and these samples correlated with a significance level of $P < 0.01$ ($r = 0.95$) (Fig. 3c).

In the exercise study haematocrit levels increased from 42.1 (1.4)% (at rest) to 44.2 (1.2)% immediately after exercise ($P < 0.05$), thereafter values decreased to 42.1 (1.2), 41.0 (1.5) and 39.3 (1.3) ($P < 0.05$ vs. rest) at 6, 20 and 68 h post-exercise respectively. The concentration of TGF-β-1 was measured in two sets of plasma samples before and at different times after uphill running, and values were corrected for changes in plasma volume estimated by the haematocrit changes. In samples handled according to the protocol designed especially for minimization of platelet degranulation, levels of TGF-β-1 tended to increase immediately after exercise [from 1102 (186) to 1296 (109) pg ml^{-1}] ($P = 0.075$) and in samples handled according to a standard protocol, TGF-β-1 levels increased significantly from 992 (49) pg ml^{-1} (at rest) to 1301 (39) pg ml^{-1} ($P < 0.05$) at the same time point (Fig. 4). No significant difference was observed at any time point between results obtained with the two protocols ($P > 0.05$).

Discussion

The considerable divergence in reported values of plasma-TGF-β-1 in normal subjects has been attributed to complications associated with the high concentration of TGF-β-1 in platelets, and even more so to differences in

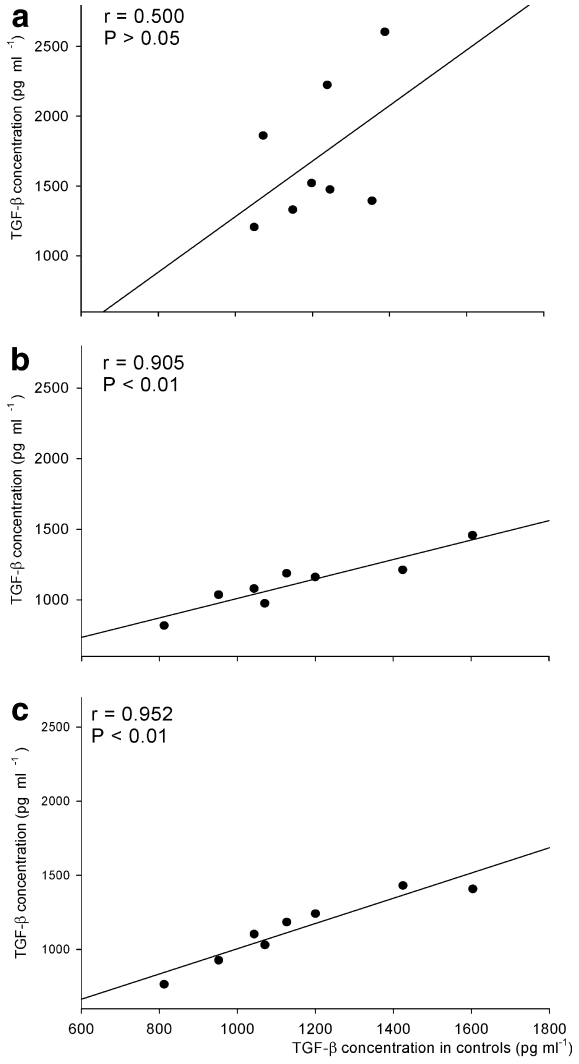

Fig. 3a–c TGF-β concentration in control samples vs. extra samples which were (**a**) not put on ice prior the initial centrifugation, (**b**) left on ice for 90 min instead of 30 min before the initial centrifugation or (**c**) sampled in EDTA tubes instead of CTAD tubes ($n=8$)

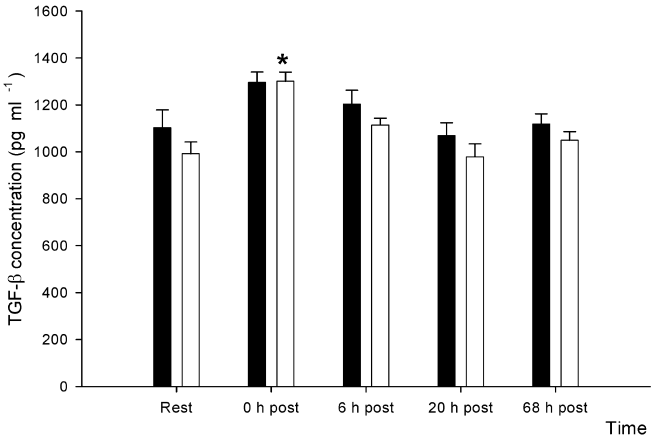

Fig. 4 Blood samples were obtained from six male subjects before and after 1 h of uphill running. The concentration of TGF-β was measured in two sets of plasma samples, which were either handled according to a protocol designed to minimize platelet activation (*filled bars*) and or according to a standard protocol for handling of plasma samples (*open bars*). Concentrations were corrected for changes in plasma volume ($n=6$). $^{*}P<0.05$

Table 2 Reported levels of plasma-TGF-β in normal subjects. Values are given as mean ± SD unless otherwise stated

TGF-β plasma concentration (pg ml^{-1})	n	Author
~500	31	(Higley et al. 1994)
~520	7	(Hering et al. 2002)
6100 (range 2900–8600)	20	(Lev et al. 2002)
1150 (30) mean (SE)	8	Present study 2003
1300 (600)	34	(Ogasawara et al. 2000)
1400 (800)	20	(Shirai et al. 1994)
1500 (200)	11	(Hayasaka et al. 1996)
2600 (1100)	12	(Murase et al. 1994)
3800 (2900)	21	(Junker et al. 1996)
4100 (2000)	42	(Wakefield et al. 1995)
4300 (300)	20	(Kong et al. 1995)
4500 (1200)	44	(Shariat et al. 2001)
6200 (1100) mean (SE)	41	(Ishitobi et al. 2000)
6500 (2300)	13	(Szymkowiak et al. 1995)
11,100 (6400	23	(Narai et al. 2002)
12,600 (1800)	16	(Suzuki et al. 2000)
18,300 (11,600)	13	(Wiercinska-Drapalo et al. 2001)
18,300 (1600) mean (SE)	13	(Flisiak et al. 2000)

the assays used for determination of TGF-β-1 concentrations (Grainger et al. 2000; Kropf et al. 1997). Large variations in plasma-TGF-β-1 levels observed with the use of identical commercial ELISA kits, e.g., the Quantikine kit from R&D Systems [values ranging from 1500 pg ml^{-1} (Hayasaka et al. 1996) to 18,300 pg ml^{-1} (Flisiak et al. 2000)], underline the significance of avoiding contamination of plasma with platelet-derived TGF-β-1. We found a stable and relatively low resting level of plasma-TGF-β-1 measured over a period of 5 days. Several precautions, partly based on observations made by Wakefield et al. (1995), were taken in order to minimize in vivo and ex vivo platelet degranulation, and this could explain why the measured plasma-TGF-β-1 levels were low compared to the majority of previously reported values (Table 2). Yet, we found that several alterations in the protocol, e.g., using K_3EDTA instead of CTAD as an anticoagulant, did not influence the measured levels of TGF-β-1. Only plasma originating from blood that was not cooled prior to separation contained elevated levels of TGF-β-1, and even in this instance the level of TGF-β-1 was comparatively low (Table 2). Furthermore, results of the exercise experiment indicated that several of the precautions taken in the blood sampling method designed especially for minimization of platelet activation were superfluous, as no difference was found between the results obtained with this method and results observed with a standard plasma sampling protocol. This indicates that K_3EDTA, which was used in the standard protocol, is a sufficient anticoagulant when measuring plasma-TGF-β-1.

However, it should be noted that cooling is seemingly more important when using K_3EDTA than CTAD, as we have measured levels of TGF-β-1 in the range of 20,000 pg ml^{-1} when blood obtained in K_3EDTA tubes was left at room temperature for 30 min prior to separation (Heinemeier 2002). In view of this, and considering the limited increase we found in plasma-TGF-β-1 from un-cooled blood samples obtained in CTAD, this anticoagulant should be preferred to EDTA, at least under circumstances where cooling is hindered. In fact, it seems likely that the use of EDTA as an anticoagulant, in parallel with insufficient cooling, could explain several of the high plasma-TGF-β-1 levels reported in the literature (Ishitobi et al. 2000; Narai et al. 2002; Suzuki et al. 2000; Wiercinska-Drapalo et al. 2001). Another important aspect of attaining reliable measures of plasma TGF-β-1 could be the method for drawing blood, and seeing that we obtained relatively low and stable levels of TGF-β-1, it is likely that the in vivo platelet activation was kept at a minimum with the method employed in this study. However, based on our results, we cannot evaluate the necessity of the precautions taken in order to diminish in vivo platelet activation, although observations made by others underline the importance of limiting the trauma induced by blood sampling (Wakefield et al. 1995).

It is possible that the relatively low levels of TGF-β-1 we observed could be connected to an incomplete or reversible separation of mature TGF-β-1 from its binding proteins during and after activation of the samples. However, the activation procedure employed in the present study (recommended by R&D Systems) has been investigated and shown to be reliable compared to other procedures (Kropf et al. 1997), and it seems unlikely that the results are biased on this account. Furthermore, we have had no complications in detecting high levels of TGF-β-1 in samples where some degree of platelet activation had taken place (Heinemeier 2002).

The present study is, to our knowledge, the first to report repeated measurements of plasma-TGF-β-1 in humans over a short time period. Over longer intervals (2 years) considerable intra-individual differences have been observed, with levels of plasma TGF-β-1 varying more than threefold in individual subjects (Wakefield et al. 1995). In contrast to this, we found a stable level of plasma-TGF-β-1 on five successive days in a group of normal subjects. Though individual variations were in some instances notable, the average CV was less than 10%, indicating that the individual plasma TGF-β-1 concentration is relatively stable over a limited period of time. This is further supported by the fact that it was possible to detect an increase in the range of 300 pg ml^{-1}, in response to 1 h of exercise in a group of only six subjects, since a more pronounced individual variation could well have masked this moderate change. These results also suggest that complications related to the detection of moderate changes in plasma TGF-β-1, e.g., in response to exercise, are not linked to a large intra-individual variations, but are more likely connected to difficulties in avoiding contamination of blood samples with platelet-derived TGF-β-1. In relation to this, Suzuki et al. (2000) found levels of 12,600 (1800) pg ml^{-1} at rest, and 13,200 (2000) pg ml^{-1} following a marathon race, but this change did not reach significance. The high basal level of TGF-β-1 observed by Suzuki and coworkers (2000) could indicate that plasma samples were contaminated with TGF-β-1 from platelet granules, especially as blood samples were not cooled in this study, and it seems likely that a moderate exercise-induced change in plasma-TGF-β-1 would easily be overlooked when measured basal levels of TGF-β-1 are in the range of 13,000 pg ml^{-1}. With regard to the proposed importance of the blood sampling procedure, it may seem strange that we, in this study, found an exercise-induced increase in plasma-TGF-β-1 in samples obtained according to a standard protocol, whereas the TGF-β-1 level in plasma sampled according to a specially designed protocol only tended to increase (Fig. 4). When considering the low number of subjects, however, and the fact that no significant difference was detected between results obtained with the two protocols, it seems likely that the discrepancy in significance level is due to random variation and not to a systematic difference caused by the blood sampling procedure.

The increase in circulating levels TGF-β-1 seen in response to exercise can have several explanations. Prolonged exercise has been shown to increase platelet count and activation (Drygas 1988), and it is possible that the exercise-induced increase found in plasma-TGF-β-1 is caused by platelet release of TGF-β-1. However, considering that TGF-β-1 is present in practically all cell types (Bonewald 1999) and that numerous in vitro studies have revealed a strong relationship between mechanical loading and TGF-β-1 synthesis in various cell types (Cillo et al. 2000; Cucina et al. 1998; O'Callaghan and Williams 2000; Skutek et al. 2001; Yasuda et al. 1996), it is likely that tissues that are mechanically loaded during exercise, including bone, muscle, tendon, cardiac, and vascular tissues, may increase their synthesis of TGF-β-1 in response to exercise and in this way contribute to an increase in the circulating TGF-β-1. Regardless of the cause, the exercise-induced increase in systemic TGF-β-1 is an interesting finding, as TGF-β-1 is known to suppress immune function (Prud'homme and Piccirillo 2000) and could thus contribute to the temporary period of immune cell functional depression that has been reported to occur after prolonged bouts of exercise (Pedersen and Hoffman-Goetz 2000).

The present study shows that the plasma-TGF-β-1 level in a group of healthy subjects is reproducible over time providing that simple precautions especially regarding cooling of blood samples are taken. Furthermore it was shown that the intra-individual variation in plasma-TGF-β-1 is moderate when subjects are at rest, and that exercise induces an increase in the concentration of TGF-β-1, possibly linked to mechanical loading of tissues during exercise.

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