Studies on the Acute Release of Tissue-Type Plasminogen Activator From Human Endothelial Cells In Vitro and in Rats In Vivo: Evidence for a Dynamic Storage Pool

By Y. van den Eijnden-Schrauwen, T. Kooistra, R.E.M. de Vries, and J.J. Emeis

The process of acute release of tissue-type plasminogen activator (tPA) is important in locally speeding up fibrinolysis. Using a sensitive enzyme-linked immunosorbent assay for tPA, we investigated the acute release of tPA from cultured human umbilical vein endothelial cells. The addition of thrombin (0.003 to 3 NIH U/mL) caused the dose-dependent release of noncomplexed, enzymatically active tPA into the medium. The amount of tPA released into the medium by thrombin was similar to the difference in the amounts of tPA present in extracts from thrombin-treated cells and control cells. The process of acute release of tPA was complete in 1 minute, whereas the concomitant release of von Willebrand factor into the medium was slightly slower (maximum after 3 minutes). By increasing (c.q. decreasing) tPA synthesis, it was found that the amount of tPA constitutively secreted, the amount acutely released, and the amount in cell extracts were increased (c.q. decreased) to the same extent. The same relation was found in vivo. When rats were pretreated with chocholate or retinoic acid to increase tPA synthesis, plasma levels of tPA were increased, whereas acute release of tPA, as induced by bradykinin, was increased to the same extent. Acutely released tPA and constitutively secreted tPA were liberated from different pathways in human umbilical vein endothelial cells; tPA had, relative to the in vivo situation, a short residence time in the acutely releasable pathway.

© 1995 by The American Society of Hematology.
(M199, containing 0.03% (wt/vol) HSA, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 2 mM/L L-glutamine) was added to the wells. Thirty minutes later, at t = 0, 15 μL human α-thrombin in M199/HSA was added to the indicated final concentration (generally 1 NIH U/mL). The same volume of M199/HSA medium was added to control cells. The media were then collected after 3 minutes, unless otherwise stated. The cells were washed once with ice-cold PBS and immediately put on ice. Cell lysates were made by scraping the cells with a rubber policeman into 0.3 mL PBS containing 0.05% (vol/vol) Tween 20, 0.01 mol/L EDTA, and 0.5% (vol/vol) Triton X-100. All incubations were performed at 37°C.

**Decreasing the constitutive secretion of tPA.** To decrease the constitutive tPA secretion, HUVECs were cultured for 0, 2, 4, 8, and 24 hours in the deficient medium described above.

**Increasing the constitutive secretion of tPA.** HUVECs were incubated for 24 hours with 0, 0.3, 1, or 3 mM/L sodium butyrate
d or with 1 μm/L RA, in total M199 medium to increase the constitutive secretion of tPA.

**Methionine-labeling of the constitutive and regulated tPA pathways.** HUVECs were cultured for 24 hours in total M199 medium, containing 3 mM/L sodium butyrate. The cells were then incubated in total M199 medium that had been made free of methionine by overnight dialysis against methionine-free M199 and that contained radiolabeled [35S]-methionine (20 μCi/well) and 3 mM/L sodium butyrate. A 100-fold excess of unlabeled methionine was added after 0, 1, 2, 3, or 4 hours. After a total incubation time of 4 hours, release was induced; media and cell extracts were prepared, and casein and EDTA were added as described. The collected media and cell extracts were incubated overnight at 4°C in microtiter plate wells, which had been coated with antibodies against tPA as described for the ELISA procedure. For controls, we used wells that had been coated with the same concentration of a monoclonal mouse antibody against FITC. After washing, the bound protein was dissolved with 0.3 mol/L NaOH, neutralized with 1.5 mol/L HCl, and counted.

**Inhibition of the constitutive and regulated tPA pathways by inhibiting protein synthesis.** To inhibit protein synthesis, HUVECs were incubated with 5 μg/mL cycloheximide in total M199 for 0, 0.5, or 1 hour. The acute release was then induced in the presence of 5 μg/mL cycloheximide (without culturing the cells for 2 hours in deficient medium). In this experiment, 2 μm/L calcium ionophore A23187 was used to induce acute release of tPA, because calcium ionophore A23187 is a receptor-independent stimulant of tPA and vWF release, which is not dependent on protein synthesis. Also, A23187 is known to induce the acute release of tPA.

**Measuring overall protein synthesis.** Overall protein synthesis was determined by incorporating [35S]-methionine into endothelial cell proteins and measuring the amount of [35S]-methionine in the trichloroacetic acid-precipitable fraction, as described.

**Induction of the acute release of tPA in vivo.** A control group of eight male Wistar rats (Iffa-Credo, Someren, the Netherlands), weighing 200 to 300 g, were fed a standard laboratory diet. To a second group of eight animals, RA (8 mg/kg body weight) was orally administered daily for 5 days to increase tPA synthesis. A third group was intravenously injected with cholecalciferol (500 μg/kg body weight) 48 hours before an experiment. Rats were anesthetized with Nembutal (60 mg/kg intraperitoneally) and were cannulated, and bradykinin (50 μg/kg body weight) was injected as a bolus into the vein of the penis. Blood was collected through a carotid artery cannula, and citrated plasma was prepared. Rat tPA antigen concentrations were determined in citrated plasma by ELISA. Animal experiments had been approved by the Animal Experiments Committee of the Netherlands Organization for Applied Scientific Research TNO and were in accordance with the guidelines on animal experimentation presented to the International Committee of Thrombosis and Haemostasis.

**Assays.** Human tPA antigen was measured by ELISA as described. Recombinant human one-chain tPA (Activase; Genetech, San Francisco, CA) was used for making calibration curves in a range of 30 to 500 pg/mL. The detection limit in this assay is 10 pg/mL. Both tPA and tPA-PAL1 complexes are detected with equal efficiency by this ELISA. Rat tPA antigen was measured by ELISA using rabbit-antirat tPA IgG, as described. 17 vWF antigen was measured by ELISA, essentially as described, with the following minor modifications: the rabbit antihuman vWF IgG was diluted 1:2,000, and 0.1% (wt/vol) of casein was used in all buffers instead of 0.05% (wt/vol) bovine serum albumin. Human pooled plasma in a range of 0.078% to 1.25% was used for calibration. Lactate dehydrogenase activity was determined using a kit, according to the manufacturer's (Sigma) instructions.

**Fibrin zymography.** Samples were put immediately in sample buffer containing 2% sodium dodecyl sulfate (SDS) and were frozen at -20°C. SDS/9% polyacrylamide slab gels were prepared according to Laemmli. Gels were then washed, placed on top of a plasminogen-rich fibrin layer, and incubated for 36 hours at 37°C, as described in detail elsewhere.

**Data presentation and units used.** The data are presented as mean ± SD of three measurements. Human tPA antigen is given as picograms or nanograms per milliliter, using Activase as standard. Rat tPA antigen is given as nanograms per milliliter, using rat L2 tPA 11 as standard. vWF antigen is given as units per milliliter, 100 U being defined as the amount of vWF antigen present in 1 mL of pooled human plasma.

**RESULTS**

**The regulated (acute) release of tPA in vitro.** The constitutive and regulated secretion of tPA was studied in vitro using first-passage human endothelial cells. In a representative experiment (Fig IA), control cells constitutively secreted 13 pg of tPA per 2 × 106 cells into the medium over 10 minutes (1.3 pg tPA/min/2 × 106 cells). This rate of constitutive tPA secretion resembled the rate of constitutive tPA secretion during the 30-minute preincubation period (1.5 pg tPA/min/2 × 106 cells) and during the 2 hours preceding the experimental period (2.2 pg tPA/min/2 × 106 cells). These data suggested that no major changes in constitutive secretion occurred because of manipulation of the cells during an experiment. On addition of human thrombin (1 NIH U/mL), the cells very rapidly released tPA into the medium. The highest increase of tPA occurred during the first minute, resulting in an additional 25 pg tPA/min/2 × 106 cells in the medium. The tPA content of the extracts from thrombin-treated cells was always lower than the content of the extracts from control cells (see Fig IA). This difference in tPA content between extracts from thrombin-treated cells and control cells was of the same magnitude as the amount released into the medium. In five experimental series, the difference averaged 94% ± 30% (mean ± SD) of the amount of tPA acutely released by thrombin into the medium at 1 minute.

Because, in vivo, acute release of vWF and tPA are often observed together, the secretion of vWF was measured in this experiment as well. vWF was, similar to tPA, constitutively secreted by control cells, 0.033 U/10 min/2 × 106 cells (see Fig IB). On addition of thrombin (1 NIH U/mL), vWF was released slightly slower into the medium than tPA. The increase in vWF was maximal at 3 minutes, at which time an additional 0.22 U/mL of vWF had been released into the medium. Figure IB also shows that the increase of vWF in the medium resulted in a corresponding decrease of vWF.
treated cells

concentrations in the cell extracts at t = 0 are low as compared with the other time points. Similarly low values have been observed more often at random time points and are not understood. In (B), the vWF concentration (expressed as U/2 \times 10^5 cells) is shown for the same experiment using the same symbols as in (A). All data shown are mean ± SD (n = 3).

cell extracts. Because the acute release of both tPA and vWF has reached maximal values at 3 minutes, in subsequent experiments, the acute release of tPA and vWF was always determined at 3 minutes. The release of tPA and vWF was not caused by cell lysis, because the lactate dehydrogenase content of media was not increased by thrombin and always remained below 3% of the amount present in the cells.

To study whether free tPA or tPA-plasminogen activator inhibitor (PAI) complex was acutely released, samples of media and cell extracts were analyzed by fibrin zymography. Despite the presence of an approximately fivefold excess of PAI-1 in these media, complex formation is prevented by an SDS-containing buffer of the media, immediately after a 3-minute stimulation. Figure 2 shows that only free tPA, but not tPA:inhibitor complex, was acutely released into the medium on addition of thrombin (Fig 2, lanes 2 and 3), and that free tPA had disappeared from the cell extracts (Fig 2, lanes 4 and 5). Please note that tPA in cell extracts has the same mobility (ie, the same M,) as constitutively secreted and acutely released tPA in media (see Fig 2, lanes 2 through 5).

Dose-dependence of tPA release by thrombin. Figure 3 shows that thrombin increased the amount of tPA (and vWF) released in a dose-dependent manner. In this experiment, the constitutive secretion of tPA was 60 pg/2 \times 10^5 cells in 30 minutes. In the presence of 1 NIH U/mL of thrombin, maximal acute release of 40 pg/2 \times 10^5 cells was observed at 3 minutes. From 0.01 NIH U/mL upwards, the acute release exceeded the constitutive secretion by at least twice the standard deviation. Similar data were obtained for vWF (Fig 3).

To see if cells could release still more tPA on further
STUDIES ON THE ACUTE RELEASE OF tPA

Fig 3. Thrombin dose-dependently induces the acute release of tPA and vWF. Concentrations of 0 to 3 NIH U/mL of thrombin were used to induce acute release of tPA and vWF. The amount of tPA (e; pg/2 x 10^5 cells) that was acutely released into the medium by the indicated concentration of thrombin and the amount of acutely released vWF (A; 10^-3 U/2 x 10^5 cells) are shown.

stimulation after maximal thrombin stimulation, cells treated with thrombin (1 NIH U/mL for 3 minutes) were subsequently incubated with calcium ionophore A23187 (1 μmol/L) for another 7 minutes. Cells treated with thrombin followed by ionophore did not release more tPA than did cells treated only with thrombin. The doubly stimulated cells released 114% ± 44% (n = 6; n.s.) of tPA, similar to the amount released by cells treated only with thrombin. Doubly stimulated cells released in 10 minutes 148% ± 73% (n = 6) of vWF, as compared with cells treated for 10 minutes with thrombin only.

Constitutive tPA secretion and acute release of tPA in various cell isolates. To investigate whether constitutive tPA secretion (representing tPA synthesis) influenced acute tPA release in HUVECs, a correlation study was performed with data from 17 isolates of HUVECs. These 17 isolates provided naturally occurring variable levels of constitutive tPA secretion (from 2 to 10 ng/mL/24 h). By linear regression analysis, a correlation between constitutive tPA secretion and acute release of tPA of 0.68 was found (Fig 4). The percentage of tPA in the cell extract that could be released averaged 33% with an SD of 17%.

Constitutive tPA secretion and acute release of tPA: Decreased constitutive secretion of tPA. When HUVECs were cultured in deficient medium, the levels of the three tPA parameters constitutive secretion, acute release, and cellular concentration (expressed as percentages of the level at t = 0) significantly decreased in parallel in time to about 20% after 24 hours (Fig 5; the absolute values of these parameters are given in the legend to the figure). These parallel changes suggest that constitutive secretion, acute release, and cellular content of tPA are closely linked by some, as yet undetermined, physiological process(es). At 24 hours, the rate of overall protein synthesis did not differ from that in control cells (127% ± 18%, n = 6), showing that the viability of cells cultured in deficient medium had not diminished. Furthermore, vWF release did not differ between cells cultured in deficient medium and cells cultured in total medium (data not shown). These data suggest that cells in deficient medium were as sensitive to thrombin stimulation as were cells cultured in total medium.

Constitutive tPA secretion and acute release of tPA: Increased constitutive secretion of tPA. HUVECs were treated with 0 to 3 mmol/L sodium butyrate or with 1 μmol/L RA to increase tPA synthesis. After 24 hours, acute release of tPA was induced. The constitutive secretion of tPA was enhanced dose-dependently by sodium butyrate, showing a 20-fold stimulation at 3 mmol/L (Fig 6). These

Fig 4. tPA synthesis and thrombin-induced acute release of tPA in 17 isolates of HUVECs. In 17 isolates of HUVECs, the acute release of tPA was induced by 1 NIH U/mL of human a-thrombin, as described in Materials and Methods. The mean constitutive tPA secretion (30 minutes) and the mean tPA release (3 minutes after thrombin-addition) are plotted; the horizontal and vertical bars represent the corresponding SDs (n = 3). After linear regression, a correlation of 0.68 was found (n = 17).
increases in constitutive tPA secretion were paralleled by increased concentrations of tPA in the cell extracts and by increased acute release of tPA (Fig 6), again suggesting that the processes involved are closely linked. RA enhanced the constitutive secretion twofold, which resulted in a 1.1-fold increase of tPA in the cell extracts and in a 1.6-fold increase of acute release of tPA. Increasing the constitutive tPA secretion did not influence the time profile of acute release, whereas, in most experiments using thrombin stimulation followed by ionophore, all available tPA was released by thrombin (data not shown). Sodium butyrate and RA did not change constitutive vWF secretion, the amount of vWF in cell extracts, or the acute release of vWF.

An acutely releasable tPA pool in HUVECs. The close correlation between constitutive tPA secretion and acutely releasable tPA, described above, might suggest that acutely releasable tPA is derived from the same pathway as constitutively secreted tPA. If so, the induction of acute release of tPA should cause a decrease in constitutive secretion over the subsequent time period. This was studied as follows: at time 0, acute release was induced with thrombin; after 3 minutes, hirudin (0.67 µg/mL) was added to the cells to inactivate thrombin; and the media were collected at 0, 3, 10, 60, and 120 minutes after the addition of thrombin. Figure 7 shows that thrombin-treated cells had released 17 pg tPA/2 × 10⁵ cells at t = 3 minutes, and that this difference persisted for the next 2 hours. These data suggested that the releasable pool was not part of the constitutive tPA pathway. In a first attempt to discriminate between tPA following the constitutively secreted one, the acute release of tPA was induced as described above, and the reaction was stopped at t = 3 minutes with hirudin. The concentration of tPA in the cell extracts was then measured after 0, 3, 10, 60, and 120 minutes. tPA is continuously secreted into the medium (a), and, on addition of thrombin, additional tPA is secreted into the medium (e), which is still found in the medium after 60 and 120 minutes. Data shown are mean ± SD (n = 3).
plates was too high to permit reliable determination of specific binding (data not shown). As shown in Fig 8, after a 1-hour chase with unlabeled methionine, the amount of radioactivity bound from media of unstimulated cells was less than 50% relative to the amount bound from unchased medium of unstimulated cells. In media from thrombin-stimulated cells, the amount of radiolabel additionally released by thrombin was still 100% relative to the amount additionally released from unchased cells. After 2 hours, both pathways contained about 25% of the amount of label present in unchased media. Therefore, these data suggest that the releasable tPA pool is separate from the constitutive secretion pathway. The inset of Fig 8 shows that comparable data are obtained when protein synthesis is inhibited with cycloheximide (overall protein synthesis, as measured by incorporation of radiolabeled methionine, was blocked for 90% after treatment of the cells for 1 hour with cycloheximide). The constitutive secretion of tPA antigen was decreased by 57% after 1 hour. In contrast, the acutely released amount of tPA antigen was still 114%, supporting the evidence for a separate intracellular source for tPA. Blocking of protein synthesis for more than 1 hour severely reduced the cellular adenosine triphosphate content (data not shown); thus, experiments were not continued beyond 1 hour.

**Acute release of tPA in vivo.** To study the relation between constitutive secretion and acute release of tPA in vivo, rats were pretreated for 2 days with cholera toxin or for 5 days with RA to increase tPA synthesis. After 2 days of cholera toxin treatment, tPA mRNA levels in heart and lung were significantly increased as were the tPA antigen concentrations, showing increased tPA synthesis in these animals (data not shown). Treatment of rats with RA also resulted in increased levels of tPA activity and tPA antigen in several tissues.

Treatment with cholera toxin increased the steady-state plasma levels of tPA 5.4-fold as compared with that for control animals (ie, t = 0 in Fig 9); treatment with RA increased tPA plasma levels 1.4-fold (Fig 9). The acute release of tPA was then induced in these animals by bradykinin. As shown in Fig 9, the acute release of tPA had, at 1 minute, increased 1.9-fold in RA-treated rats and 3.7-fold in cholera toxin-treated animals, as compared with that for control animals. These data indicate that an increased tPA synthesis gives rise to an increased acute release of tPA in vivo as well.

**DISCUSSION**

In this study, we addressed the questions of how acute release of tPA from human endothelial cells proceeds and how this release is related to the constitutive secretion of tPA. Using first-passage HUVECs, we observed, as have many others, constitutive secretion of tPA into the medium at a steady rate over many hours. On the addition of thrombin, acute release occurred, as evidenced by the rapid appearance of uncomplexed, enzymatically-active tPA in the medium. The acute release of tPA was very rapid, showing a maximal increase of tPA within 1 minute. In both these respects, the acute release reaction in vitro resembled acute release of tPA as observed in humans in vivo. Release induced by thrombin was dose-dependent. At maximal stimulation by thrombin (>1 NIH U/mL), most if not all endothelial tPA was released into the medium, because a second
stimulation by calcium-ionophore did not result in significantly more tPA release. Concomitantly with tPA, vWF was also released, as has often, but not always, been observed in vivo.18,22 Compared with tPA, vWF was released slightly more slowly, showing a maximum at 3 minutes. The time course of the acute release of tPA and vWF in vitro also resembled the time course of acute release in the perfused rat hindleg, which also showed very rapid release of tPA resembling the time course of acute release in the perfused rat hindleg. The highest concentration of tPA is found at 1 minute. After 1 minute, the tPA concentration decreases because of clearance by the liver. The vertical bars represent SDs of the mean (n = 8).

The amount of thrombin-releasable tPA strongly correlated with its rate of constitutive secretion (ie, tPA synthesis) in HUVECs (Figs 4 through 6). A similar correlation was found in vivo in rats (Fig 9). Because the constitutive secretion of tPA (tPA synthesis) was coupled, at least quantitatively, so closely with the release of tPA, the question arose how closely the two pathways are coupled functionally. The following evidence suggests that both pathways are not identical. Thrombin-induced release of tPA does not diminish the subsequent constitutive secretion (Fig 7), indicating that the former is not a precursor of the latter. After metabolic labeling (Fig 8), released tPA retains label about 1 hour longer than constitutively secreted tPA. Also, after inhibition of protein synthesis by cycloheximide, release of tPA is maintained for at least 60 minutes at control level, whereas the level of constitutively secreted tPA decreases strongly. These observations suggest that releasable tPA was delayed in an additional compartment. This conclusion is supported by the observation that acutely released tPA is not complexed to an inhibitor (Fig 2). Because tPA in the subcellular matrix and tPA on the plasma membrane are complexed to PAI-1,23 the tPA released must be derived from an intracellular compartment (see also Fig 2, lane 4), in line with previously published data,10 which showed that plasma membrane-bound tPA is not released. Moreover, on subcellular fractionation,23 the tPA content of an intracellular high-density fraction, free of plasma membrane markers, is diminished after stimulation with thrombin (Schrauwen et al19 and Emeis and van den Eijnden-Schrauwen, manuscript in preparation). Therefore, we propose that, after synthesis, tPA is temporarily stored in an intracellular compartment, presumably consisting of high-density granules. In all these respects, the pathway followed by tPA destined for acute secretion resembles that of other secretory proteins,6,24 although the storage pool is less permanent than pools of other stored proteins.

We observed a close correlation between the rate of synthesis of tPA and the amount of acutely releasable tPA. This suggests that a fixed percentage of newly synthethized tPA goes into the tPA storage compartment, regardless of the rate of synthesis. This has also been described for vWF storage.25,26,27 tPA that is not acutely released from this pool into the medium still disappears from the pool, because the time tPA stays in the pool is only about 1 hour. What happens to this tPA is not known. We call this tPA that has disappeared "lost tPA," because it is not secreted in the constitutive pathway (Fig 7) or in the acute release pathway. One possibility is that this lost tPA is deposited in the subcellular matrix.28 However, we have been unable to detect the required amounts of tPA in the matrices of our cell cultures. Another possibility is that the lost tPA is degraded intracellularly, possibly in a lysosomal compartment, because label leaves the storage compartment but is not recovered in the constitutive pathway (Fig 8; compare also Fig 7). In an attempt to find further evidence for this hypothesis, we incubated cells for 4 hours in conditions that diminish lysosomal enzyme activity (50 µmol/L chloroquine). Under these conditions, the constitutive secretion did not change, whereas the amount of tPA in cell extracts did indeed increase. However, this increase could account for only some 10% of the lost tPA (our unpublished observations).

The storage pool in HUVECs (residence time about 1 hour) was less stable than the tPA storage pool in vivo in rats, the latter pool being hardly diminished by inhibiting protein synthesis for 5 hours.29 This might explain why endothelial cells in vitro possess a relatively small storage pool for tPA as compared with that for rats. In cell extracts from HUVECs about 0.1 ng of tPA/cm² is present, whereas 0.5 to 2 ng of tPA/cm² has been estimated to be present in vivo (calculations in Emeis28).

Acutely released tPA may play an important role in speeding up thrombolysis. Not only is the tPA thus released itself enzymatically active, but acute release will also result in a local tPA concentration much larger than the basal level of tPA and also that of its inhibitor PAI-1. Thrombin, which is an effective stimulus for the induction of tPA release (Fig 3), may be the major stimulus for tPA release, because it will reach high local concentrations near forming
or growing thrombi. Indeed, in vivo in primates, thrombin formation has been shown to induce acute release of large amounts of tPA. In quantitative terms, acute secretion of 50 pg tPA/cm² (as in Fig 6) will result, in a capillary with a diameter of 5 μm, in a local concentration of 340 ng tPA/mL. If tPA synthesis is increased (Fig 6), up to 900 pg tPA/cm² can be released, resulting in a local concentration of 6 μg tPA/mL in that same capillary (compare with Schrauwen et al). This concentration is even higher than the plasma tPA concentration reached during thrombolytic therapy. Moreover, tPA present before or during coagulation will be much more potent in dissolving a thrombus than tPA present only after thrombus formation has occurred, as is the case during thrombolytic therapy.

The results of this study show that in human endothelial cells tPA can be acutely released from an intracellular pool, and that it should be possible to enhance this capacity for acute tPA release by increasing tPA synthesis. Therefore, in combination with the possibility to induce the release of tPA without inducing the release of vWF, it should be possible to increase local tPA concentrations specifically and to speed up fibrinolysis/thrombolysis when required.

REFERENCES
1. Brommer EJP: The level of extrinsic plasminogen activator (t-PA) during clotting as a determinant of the rate of fibrinolysis; inefficiency of activators added afterwards. Thromb Res 34:109, 1984
3. Schrauwen Y, de Vries REM, Kooistra T, Emeis JJ: Acute release of tissue-type plasminogen activator (tPA) from the endothelium; regulatory mechanisms and therapeutic target. Fibrinolysis 8:8, 1994 (suppl 2)
6. Emeis JJ: Regulation of the acute release of tissue-type plasminogen activator from the endothelium by coagulation activation products. Ann NY Acad Sci 667:249, 1992
Studies on the acute release of tissue-type plasminogen activator from human endothelial cells in vitro and in rats in vivo: evidence for a dynamic storage pool

Y van den Eijnden-Schrauwen, T Kooistra, RE de Vries and JJ Emeis