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The misleading nature of *in vitro* and *ex-vivo* findings in studying the impact of stress hormones on NK cell cytotoxicity

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Abstract

In vitro and *ex-vivo* studies assessing the impact of stress hormones on immune competence commonly replace the natural milieu of leukocytes with an artificial medium, excluding plasma factors, hormones, and cytokines. Given prevalent inconsistencies between *in vitro*, *ex-vivo*, and *in vivo* findings, we studied whether such procedures could yield misleading outcomes regarding the impact of stress hormones on NK cell cytotoxicity (NKCC), using fresh human whole blood samples. We found that in the presence of plasma 10-30-fold higher concentrations of cortisol, epinephrine, and prostaglandin-E2 (PGE2) were required to reach suppression levels evident in the context of artificial medium. Importantly, whereas the NK suppressive effects of PGE2 occurred immediately and remained stable upon prolonged exposure, the suppressive effects of cortisol slowly increased over time. Last, to simulate the exclusion of stress factors in the *ex-vivo* approach, we subjected whole blood to stress hormones (as occurs *in vivo*), and abruptly removed them. We found that the effects of epinephrine and PGE2 quickly disappeared, while the effects of cortisol persisted. Overall, these findings demonstrate the potential misleading nature of *in vitro* and *ex-vivo* procedures, and specifically suggest that (i) the common *in vitro* findings of profound suppression of NKCC by stress hormones are overestimation of their direct effects expected *in vivo*; and (ii) the common *ex-vivo* approach cannot reflect the direct *in vivo* suppressive effects of epinephrine and PGE2 on NKCC, while inflating the effects of glucocorticoids. Some of these fallacies may be circumvented by using non-delayed whole blood NKCC assays in humans.

Keywords

NK cytotoxicity; cortisol; prostaglandin; epinephrine; in vitro; ex-vivo; in vivo; stress; human; whole blood

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1. Introduction

Stress and stress hormones are known to alter the function of Natural Killer (NK) cells. However, significant inconsistencies are prevalent between *in vitro*, *ex-vivo*, and *in vivo* findings regarding the nature and direction of the effects of specific stress hormones or stress paradigms on NK cell cytotoxicity (NKCC) [1]. For example, epinephrine was reported to suppress NKCC *in vitro*, both in human and animal blood [2–5], through activating NK cell adrenergic receptors and the consequent increase in intracellular cAMP levels [3]. However, *ex-vivo* human and animal studies reported contradictory results; many have demonstrated that administration of epinephrine, acute stress exposure, or exercise enhances NKCC [6–11], whereas some have reported suppression of NKCC [12–14]. Animal studies employing *in vivo* procedures generally inferred a suppressive effect of epinephrine on NK activity [12, 15–17].

Glucocorticoids in physiological concentrations were repeatedly shown to markedly suppress human and animal NKCC *in vitro* [18–20]. However, several *ex-vivo* studies in humans and animals have suggested that no such suppression occurs *in vivo* [21, 22], and a recent *in vivo* animal study has supported this suggestion, indicating specific conditions under which corticosterone may exerts some effects [16].

Unlike catecholamines and glucocorticoids, the release of prostaglandins (PGs) is not controlled centrally. Rather, PGs are released locally by a variety of cells [23, 24], including malignant cells [25], and as a response to tissue damage [26]. Under some conditions (e.g., surgery) local release can markedly increase systemic PGs levels [27, 28]. *In vitro* studies showed that prostaglandin-E2 (PGE2) can markedly suppresses NK activity [19, 29, 30], and *in vivo* studies reported deleterious impacts of PGs on resistance to cancer metastases [22], which is allegedly mediated through *in vivo* suppression of NK cells [31]. However, in a recent study in rats, we have provided evidence indicating that a direct *in vivo* suppressive effect of PGE2 on NKCC cannot be evident in an *ex-vivo* assessment of NKCC [5]

We hypothesize that most inconsistencies regarding the impact of stress hormones on NKCC originate from methodological obstacles and specific procedures that yield misleading outcomes. These procedures include: (1) exclusion or distortion of the natural milieu when conducting *ex-vivo* or *in vitro* testing, such as replacement of plasma with a hormone- and cytokine-free artificial medium, or testing cytotoxicity in purified NK cells; (2) overlooking the kinetics of the effects of a hormone in its presence and following its exclusion; (3) disregarding the *in vivo* effects of a stress hormone on NK cell trafficking, which may manifest itself as a change in function; and (4) disregarding the existence of different NK cell subpopulations with different cytotoxicity capacity, in conjunction with stress-induced redistribution of NK cells that is subpopulation-specific (specifically in *ex-vivo* studies). Some alleged inconsistencies result from differences in stress paradigms or hormone levels/concentrations, which we do not consider as inconsistent findings, but rather as reflecting the complexity of the effects of stress.

Although the impact of stress on immune competence should ideally be studied *in vivo*, in humans this approach is commonly impractical. Thus, it is important to elucidate the degree

to which the standard *in vitro* and *ex-vivo* approaches, used in human studies of NKCC, would reflect *in vivo* outcomes. It would be instrumental to point at specific distortions caused by these approaches, if exist. To start addressing these issues in humans, we herein simulated several critical procedural aspects of the standard *in vitro* and *ex-vivo* approaches, employing fresh whole human blood. Admittedly, such an *in vitro* study may seem limited and paradoxical in examining potential limitations of *in vitro* and *ex-vivo* approaches. Thus, we restricted the scope of the study to the assessment of aspects that can be simulated or examined by this methodology, aiming at identifying inherent impediments in standard *in vitro* and *ex-vivo* approaches. Specifically, we address the potential effects of (i) studying human NKCC in the absence of plasma factors, and (ii) disregarding the kinetics of the impact of stress hormones and of their removal from the assay medium. We address these issues employing three different putative immunemodulating stress hormones acting through different cellular or intracellular mechanisms, namely epinephrine, PGE2, and cortisol.

2. Materials and Methods

2.1. Subjects and their baseline stress levels

In all experiments, all manipulations were conducted within subject. In each experiment, 6–12 healthy volunteers (Mean age 33.07, SD 7.60) provided morning blood samples. Healthy subjects were recruited by an advertisement posted at the Tel Aviv University campus, offering financial compensation for their time. Exclusion criteria were acute sickness during the last week, alcoholism, and drug abuse. Females constituted 25% of all subjects. The study was approved by the Institutional Review Board (Helsinki committee) of Sheba Medical Center, and all participants gave written informed consent before donating their blood.

All subjects have donated blood several times in different circumstances, and were made familiar with the experimental setting of blood withdrawal. Most contributed blood for several of the 8 experiments reported herein. Before blood withdrawal, subjects were allowed to rest, and filled a questionnaire regarding their health status, medication usage, caffeine intake, exercise habits, and existing stress levels. Specifically, subjects rated their current stress levels between 1 and 10 – 1 being the lowest possible stress level ("not at all") and 10 the highest possible stress level ("extremely stressed").

2.2. Blood withdrawal and use

Venous blood (between 40–100 ml, depending on specific experiment) was collected from each subject between 8:00 and 10:00 A.M. into heparinized syringes (30 U of preservative-free heparin per ml blood). Blood samples were immediately subjected to the procedures described below.

2.3. Serum cortisol levels

Serum cortisol levels were assessed employing an ELISA kit (MP Biomedicals, Orangeburg, NY) according to manufacture instruction.

2.4. Drugs

The concentrations of the hormones used in the study cover their physiological systemic levels. For epinephrine and PGE2 higher concentrations were also used, as these hormones, but not cortisol, also interact locally with NK cells at higher concentrations – catecholamines at immunological sympathetic synapses [32, 33] and PGE2 at areas of tissue damage or malignant tissue that release PGs [26, 34].

Cortisol (hydrocortisone-Water Soluble), and epinephrine (both from Sigma–Aldrich, Israel) were dissolved in complete medium. Prostaglandin-E2 (ENCO, Israel) was dissolved in ethanol and diluted in PBS, then further diluted in complete medium, to reach a final concentration of 0.003% or less ethanol in the assay medium. In previous studies we found that a 30-fold higher concentration of ethanol is needed to start impacting NK cytotoxicity *in vitro* [35].

2.5. Complete Medium (CM)

CM is RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum (FCS), 50 μg/mL of gentamicin, 2 mM of l-glutamine, 0.1 mM of nonessential amino-acids, and 1 mM of sodium pyruvate. All compounds were purchased from Biological Industries, Kibbutz Beit Haemek, Israel.

2.6. Maintenance and radiolabeling of K562 target cells

The standard K562 tumor cell line (NK-sensitive erythromyeloid) [36] was used as target cells in the NK cytotoxicity assay. Cells were grown in CM at 37 °C in 5% CO_2. For radiolabeling, 25×10^6 cells were incubated for 1 hr with 200 μCi of ^{51}Cr (Rotem Tassiot, Dimona, Israel) in 200 μl FCS, and 100 μl CM. After incubation, cells were washed three times with CM (335g, for 10 min).

2.7. NK cytotoxicity assay

A whole blood 4h ^{51}Cr release assay was used. This procedure assesses anti-tumor NKCC without excluding any cell subtype, and potentially without excluding plasma factors. Earlier studies have indicated that cytotoxicity measured using this procedure is attributable to NK cells, rather than other cell types or soluble factors, as the selective depletion of NK cells abolishes all target-cell killing [37–39]. The advantages of this procedure include shorter preparation time, less interference with the effector cells, and better representation of the original *in vivo* milieu and cell composition.

Aliquots of 220μl blood from each subject were placed in a 96-well microtiter plate. In all studies, each plate contained blood from one subject, subjected to drug treatments (in up to seven columns) and triplicates of control (vehicle) conditions. The outcomes from the three controls conditions were averaged, and drug effects were expressed as percent of averaged control levels within plate.

To minimize dilution of plasma, 40μl of tumor cells and 20μl of drug were added to the 220μl of blood. To determine spontaneous and maximal ^{51}Cr release, blood was substituted with CM or Triton 100 (Sigma, Jerusalem, Israel), respectively. To form six E:T

(Effector:Target) ratios, 2×10^5 K562 cells were used for the lowest E:T ratio, and half the number of tumor cells was used for each of the consecutive 5 E:T ratios. The plates were centrifuged at 596g for 10 min and were then incubated for 4 h in 5% CO_2 at 37 °C. Following incubation, plates were centrifuged again at 4 °C, and 80μl of the supernatant were recovered from each well for assessment of radioactivity in a γ-counter.

Each experiment was conducted in several replications, employing different subjects. In each replication, blood samples were subjected to all treatments. However, in some experiments/replications the highest or lowest non-physiological doses were tested only in some subjects, and thus in these doses the sample sizes ("n") are smaller than in theft physiological doses.

2.8. Data analysis

2.8.1. NK cytotoxicity—To evaluate percent-specific lysis, the following formula was used: (experimental release × correction - spontaneous release)/(maximal release - spontaneous release) × 100, where correction is the percentage of the supernatant within the volume of the well (i.e., excluding the hematocrit volume). This correction is necessary because chromium released by labeled target cells is found only in the supernatant above the red blood cells [38].

2.8.2. Lytic units 45 (LU_{45})—The LU approach transforms the cytotoxicity levels evident in the different E:T ratios (6 in our study), to a single index, indicating the number of aliquots/units contained in the sample tested for cytotoxicity that can each exert a designated level of cytotoxicity (e.g., 39% specific lysis in Fig 1A). In this approach, the comparison of different conditions is based on the horizontal shift between the different cytotoxicity killing curves, rather than the vertical difference [40]. In the current study, we defined and quantified LU_{45} within each microtiter plate as the number of LU at a 45% increase between the minimal and maximal cytotoxicity in the control condition (i.e., in Fig. 1A – 45% increase from 15% to 69% specific lysis designate "39% specific lysis" as the levels for calculating LU for all cytotoxicity curves in this plate). The regression exponential fit method was used to calculate LU for each condition, based on the six E:T ratios [40].

2.8.3. Statistical analysis—Throughout the study, drug effects are expressed as percent of control levels within each microtiter plate, and thus the control condition is always at 100%, with deviations stemming from duplicates or triplicates of the control condition. Each plate always contained blood from one type of blood preparation (e.g., presence or absence of plasma) and from a single subject. This approach overcomes individual differences in baseline levels of NKCC, and enables focusing on the effects of the drugs in each of the different blood preparations. Two- or three-way analyses of variance (ANOVA) with a predetermined significance level of 0.05 were conducted to test for main effects and interactions. Provided significant group differences were found, Fisher's protected least significant differences (Fisher's PLSD) contrasts were performed to compare specific pairs of groups, based on *a priori* hypotheses. We chose not to conduct repeated measure ANOVAs, as the use of the "% of control" index within each plate/subject already addresses individual differences, and the qualitatively different preparations of the blood, used within

a subject blood sample, questions the appropriateness of using a within subject analysis. The between-subject analyses that we have conducted are more conservative.

3. Procedures and results

3.1. Subjects exhibit low stress levels at blood withdrawal

3.1.1. Self-report—All subjects reported low levels of stress at the time of blood withdrawal. The average score reported was 1.86 (SD 0.56) on a scale of 1 to 10, with a median of 2.

3.1.2. Serum cortisol levels—The average serum cortisol level in the blood withdrawn from subjects was $3.7*10^{-7}$ M (SD $1.7*10^{-7}$), with a median of $3.1*10^{-7}$ M.

3.2. Experiment 1: Markedly higher suppression of NKCC is induced in the absence of plasma compared to its presence by cortisol, PGE2, and epinephrine

This study was designed to compare whole blood (containing subjects' original plasma) to blood without plasma (replaced with CM, as in the case of the standard *in vitro* approach) with respect to the acute effects of different concentrations of stress hormones on NKCC. Thus, in this experiment, the stress hormones were applied simultaneously with the initiation of the cytotoxicity assay, while in experiments 2 and 3 the stress hormones were also applied at different time intervals prior to the cytotoxicity assay.

3.2.1. Procedure—Blood was collected into heparinized syringes. Half of the blood was washed and plasma was replaced with CM (plasma absence), and the remaining blood was kept at room temperature (plasma presence). For plasma removal, aliquots of 3 ml of blood were washed once with 12 ml of PBS and twice with 12 ml of CM (centrifuged at 596g for 10 min, and reconstituted to their original volume each wash). Following blood wash, aliquots from each subject were pooled together, and blood samples were immediately co-incubated with different concentrations of cortisol, PGE2, epinephrine, or with vehicle, as seen in Fig. 1 B–D, which cover the physiological ranges of plasma levels of these hormones. Immediately after hormones were introduced into the blood, NKCC was assessed in their presence. The study was conducted in four replicates, employing different subjects, and each replicate included all experimental conditions.

3.2.2. Results—A representative E:T cytotoxicity curve is presented for a single plate, including two replicates of control condition and 4 doses of PGE2, in the absence of plasma. (Fig. 1 A). Each curve was expressed as a single LU_{45} value, which served as the dependent value in all statistical analyses.

The four replicates of the study yielded similar patterns of results and were combined based on percent of control levels in each plate (see Materials and Methods, *2.8.3. Statistical analysis*). For each drug, an 8×2 ANOVA was conducted (8 drug concentrations including vehicle, by plasma presence/absence) with percent of averaged control levels as the dependent variable. By and large, in the presence of plasma markedly higher doses of each of the drugs were needed to cause NKCC suppression, compared to similar effects in the absence of plasma, as detailed below.

3.2 .2.1. Cortisol: A two-way ANOVA revealed main effects for cortisol concentrations ($F_{(7,156)} = 12.404$, $p < 0.0001$) and for plasma presence (presence vs. absence of plasma) ($F_{(1,156)} = 34.325$, $p < 0.0001$), and a significant interaction ($F_{(7,156)} = 5.887$, $p < 0.0001$). Specifically, cortisol dose-dependently suppressed NKCC, but this suppression was evident only in the absence of plasma. Fisher's PLSD indicated that in the absence of plasma cortisol concentrations of 3×10^{-6}, 10^{-6}, 3×10^{-7} and 10^{-7} M significantly reduced NKCC compared to the control ($p < 0.0001$), and compared to the presence of plasma ($p < 0.03$ or smaller). No significant effects were evident in any of the concentrations used in the presence of plasma (Fig. 1 B).

3.2.2.2. PGE2: A two-way ANOVA revealed a main effect for PGE2 concentration ($F_{(7,164)} = 120.37$, $p < 0.0001$) and for plasma presence ($F_{(1,164)} = 101.207$, $p < 0.0001$), and a significant interaction ($F_{(7,164)} = 40.473$, $p < 0.0001$). Specifically, PGE2 dose-dependently suppressed NKCC, but this suppression was markedly more profound in the absence of plasma. In fact, more than 30-fold higher concentrations of PGE2 were needed in the presence of plasma to reach similar levels of suppression evident in its absence. Fisher's PLSD indicated that PGE2 concentrations of 3×10^{-7}, 10^{-7}, 3×10^{-8} and 10^{-8} M significantly reduced NKCC in the absence of plasma more than in its presence ($p < 0.0001$). Additionally, concentrations of 3×10^{-7}, 10^{-7}, 3×10^{-8} and 10^{-8} M significantly reduced NKCC both in the absence and in the presence of plasma compared to the relevant control condition ($p < 0.0001$, and $p < 0.02$ or smaller, respectively) (Fig. 1 C).

3.2.2.3. Epinephrine: A two-way ANOVA revealed a main effect for concentration ($F_{(7,160)} = 110.338$, $p < 0.0001$) and for plasma presence ($F_{(1,160)} = 11.459$, $p < 0.001$), and a significant interaction ($F_{(7,160)} = 6.821$, $p < 0.0001$). Specifically, epinephrine dose-dependently suppressed NKCC, but this suppression was markedly more profound in the absence of plasma, as more than 10-fold higher concentrations of epinephrine were needed in the presence of plasma to reach similar levels of suppression evident in its absence. Fisher's PLSD indicated that epinephrine concentrations of 3×10^{-7}, 10^{-7}, and 3×10^{-8} M significantly reduced NKCC in the absence of plasma more than in its presence ($p < 0.01$). Additionally, concentrations of 3×10^{-7}, 10^{-7}, 3×10^{-8} and 10^{-8} M significantly reduced NKCC both in the absence and in the presence of plasma compared to the relevant control condition ($p < 0.0001$, and $p < 0.01$ or smaller, respectively) (Fig. 1 D).

3.3. Experiment 2: Suppression of NKCC by cortisol increases with time, whereas suppression induced by PGE2 occurs immediately and remains stable

This study was conducted to examine potential time-dependent effects of different stress hormones on NKCC, in the presence of plasma and in its absence. In this experiment, the stress hormones were introduced (i) simultaneously with the initiation of the cytotoxicity assay (0 h), or (ii) 2 h, or (iii) 6 h prior to the cytotoxicity assay. In all three conditions stress hormones remained present throughout the cytotoxicity assay.

Whereas in Exp. 1 NKCC assessment was performed shortly after blood was withdrawn, in Exp. 2 all blood samples were maintained for 6 hours prior to NKCC assessment (to enable the 6 pre-incubation period).

In this experiment, different mechanisms of action are represented through cortisol and PGE2. Cortisol was chosen due to its activation of a nuclear transcriptional mechanism [41], whereas PGE2 activates an immediate cytoplasmatic cAMP-dependent mechanism [42]. Epinephrine and PGs act through the same intracellular mechanism to suppress NKCC [3], but the effects of PGE2 were more potent at physiological levels than of epinephrine. Thus, and given limited amount of blood available per participant (100 ml), PGE2 was favored over epinephrine for this experiment.

3.3.1. Procedure—Blood was collected into heparinized syringes at a single time point. Half of the blood was washed and plasma was replaced while the remaining whole blood was kept at room temperature. For plasma removal, aliquots of 3 ml of blood were washed and centrifuged (room temperature, 596g for 10 min), once with 12 ml of PBS and twice with 12 ml of complete medium, and reconstituted to its original volume. Following blood wash, aliquots from each subject were pooled together.

Blood samples were co-incubated with different concentrations of cortisol, PGE2, or vehicle, as seen in Fig. 2, which cover the physiological ranges of plasma levels of these hormones. Hormones were first introduced to the 6 h co-incubation condition. Thereafter, all plates were kept in the incubator (5% CO_2 at 37 °C). 4 hours later hormones were introduced to the 2 h co-incubation condition and placed back in the incubator. 2 hours later hormones were introduced to the 0 h condition (i.e., 6 hours following the introduction of hormones to the 6 h condition), and NKCC was assessed for all conditions/time intervals in the presence of the hormones. The study was conducted in two replicates, employing different subjects.

3.3.2 Results—The two runs of the study yielded similar patterns of results and were combined based on percent of control levels in each plate (see Materials and Methods, *2.8.3. Statistical analysis*). As the main focus of the study was to compare the time-dependent effects between the different drugs, a 2×4×3 ANOVA was conducted separately for the replacement of plasma and its presence (2 drugs, 4 concentrations, 3 time intervals), with percent of averaged control levels as dependent variable.

3.3.2.1. Plasma absence: A three-way ANOVA revealed main effects for drug ($F_{(1,84)}$ = 8.886, $p < 0.005$), concentration ($F_{(3,84)}$ = 18.839, $p < 0.0001$), and time ($F_{(2,84)}$ = 18.051, $p < 0.0001$), and a significant drug by time interaction ($F_{(2,84)}$ = 13.797, $p < 0.0001$), indicating that PGE2 and cortisol exert significantly different time-dependent NK suppressive effects. Specifically, Fisher's PLSD indicated that cortisol significantly reduced NKCC in 6 h more than in 0 h (in concentration of 3×10^{-6}, 10^{-6}, and 3×10^{-7} M, $p <$ 0.003 or smaller), whereas PGE2 exerted the same levels of suppression at all time-intervals tested (Fig. 2).

3.3.2.2. Plasma presence: Similar effects were evident in the presence of plasma as described above in the absence of plasma, except that the suppression of NKCC by cortisol was markedly lower in the presence of plasma, as expected. A three-way ANOVA revealed main effects for drug ($F_{(1,84)}$ = 55.187, $p < 0.0001$), concentration ($F_{(3,84)}$ = 25.021, $p <$ 0.0001), and time ($F_{(2,84)}$ = 6.638, $p < 0.003$), and significant interactions between drug by

concentration ($F(_3,84) = 12.750$, $p < 0.0001$) and drug by time ($F_{(2,84)} = 4.409$, $p < 0.03$), the latter indicating that PGE2 and cortisol exert significantly different time-dependent NK suppressive effects. Specifically, Fisher's PLSD indicated that cortisol significantly reduced NKCC in 6 h more than in 0 h (in concentration of 3×10^{-6}, 10^{-6}, and 3×10^{-7} M, $p < 0.05$ or smaller), whereas PGE2 exerted the same levels of suppression at all time-intervals tested (data not shown).

3.4. Experiment 3: The effects of PGE2 and epinephrine on NKCC quickly disappear following their removal, while the effects of cortisol persist

As the standard *ex-vivo* approach incorporates the replacement of plasma by an artificial medium, and thus also the exclusion of stress hormones, in the current experiment we studied the kinetics of the effects of stress hormones on NKCC following their removal. Specifically, to simulate the *in vivo* impact of the hormones, whole blood (containing plasma) was maintained in the incubator for 2 h while subjected to different stress hormones. Thereafter, the hormones were either (i) maintained in the assay milieu while testing NKCC (hormone presence), (ii) washed away and blood immediately tested for NKCC (hormone exclusion), or (iii) washed away and blood maintained at room temperature for 4 h before testing for NKCC (hormone exclusions + 4 h). The last two conditions aimed to simulate the standard *ex-vivo* exclusion of hormones and plasma before testing for NKCC.

3.4.1. Procedure—Blood was collected into heparinized syringes, and aliquots of whole blood (containing subjects' plasma) were co-incubated for 2 hours with different concentrations of cortisol, PGE2, epinephrine, or vehicle, as seen in Fig. 3 A (covering the physiological ranges of plasma levels of these hormones). Additionally, a combination of cortisol and epinephrine, each in an effective concentration, was studied, as seen in Fig 3 B. NKCC was assessed either in the presence of these hormones, immediately after they were washed away, or 4 h after they were washed away. Except for the initial 2 hours of co-incubation with hormones, blood was processed and maintained at room temperature until assessment of NKCC. For hormone exclusion, aliquots of 1.6 ml of blood were washed twice with 3.5 ml of PBS while reestablishing volume of 200μl above hematocrit levels, and twice with 3 ml of complete medium while reconstituting to original volume of 1.6 ml (centrifuged at 596g for 10 min each time). This procedure diluted the original hormone concentrations by 7,600-fold. The study was conducted in two replicates, employing different subjects, each replicate including all experimental conditions.

3.4.2 Results—The two replicates of the study yielded similar patterns of results and were combined based on percent of control levels in each replicate (see Materials and Methods, *2.8.3. Statistical analysis*). A 3×3×3 ANOVA was conducted (3 drugs, 3 concentrations, 3 hormone impact approaches), with percent of averaged control levels as dependent variable (Fig. 3 A). In addition, a 3×3 ANOVA was conducted (3 drugs, 3 hormone impact approaches) in order to compare the individual effects of cortisol and epinephrine to their combined impact on NKCC employing a single concentration of each drug (Fig. 3 B).

3.4.2.1. Effects of cortisol, epinephrine, and PGE2: A three-way ANOVA revealed main effects for concentration ($F_{(2, 117)} = 14.466$, $p < 0.0001$), and hormone impact approaches ($F(2,_{117}) = 27.745$, $p < 0.0001$), and significant interactions for drug by hormone impact approach ($F_{(4,117)} = 11.297$, $p < 0.0001$) and concentration by hormone impact approaches ($F(_4,117) = 2.730$, $p < 0.05$). Importantly, Fisher's PLSD indicated that epinephrine and PGE2 suppressed NKCC only when they were present in the *in vitro* setting, and their effects disappeared immediately following their removal ($p < 0.05$ or smaller), whereas suppression levels of cortisol did not change following its removal as seen in Fig 3 A.

3.4.2.2. Effects of cortisol, epinephrine, and their combination: A two-way ANOVA revealed a main effect for drug ($F_{(3,120)} = 21.354$, $p < 0.0001$), with no significant interaction. Fisher's PLSD indicated that in the presence of these hormones, the combination of cortisol and epinephrine yielded significantly greater suppression of NKCC than each of these hormones individually ($p < 0.03$ or smaller), exhibiting an additive effect of these hormones. However, following the exclusion of the hormones and 4 hours later, the combination of cortisol and epinephrine suppressed NKCC similarly to cortisol alone, and significantly more profoundly than epinephrine alone, where the effect disappeared ($p < 0.05$ or smaller) (Fig. 3 B).

4. Discussion

The present study suggests that the common findings of *in vitro* suppression of NKCC by stress hormones are an overestimation of the effects expected *in vivo*. Specifically, in the presence of plasma, 10–30 fold higher concentrations of stress hormones were needed to reach the same levels of NKCC suppression evident in artificial medium (absence of plasma), which is the standard *in vitro* milieu (e.g., RPMI-1640 + various additives + 10% FCS). Moreover, at acute exposure to systemic physiological levels, minute or no effects of stress hormones were evident in the presence of plasma, while marked suppression was evident in artificial medium. It should be noted that when blood was drawn, plasma cortisol levels were at low physiological levels (~$3*10^{-7}$ M), and thus subjecting these blood samples to high physiological concentrations (10^{-6} to $3*10^{-6}$ M) constitutes a remarkable increase in cortisol levels, which, nevertheless, had no immediate effect on NKCC in the presence of plasma. These findings question the notion that acute exposure to physiological levels of stress hormones (e.g., up to 60 min of a stress paradigm), especially cortisol, directly suppress circulating NKCC *in vivo* on a per NK cell basis. Thus, we suggest that when *ex-vivo* studies report suppression of NKCC by acute stress exposure, such findings may primarily reflect redistribution effects induced by stress hormones [12], such as an influx of NK cells of an a priori lower cytotoxicity levels into the circulation.

On the other hand, under different conditions stress hormones may in fact impact NKCC on a per NK cell basis. Specifically, this may occur through a more prolonged exposure, which was evident herein to exert a direct *in vitro* NK suppressive effects in the presence of plasma, and was reported by *in vivo* studies [16]. Additionally, suppression of NKCC may occur through (i) indirect effects of stress hormones on NKCC, such as through reduction in Th1 cytokine levels [43], (ii) through direct suppression of NKCC by catecholamines and PGs when locally released at supra-systemic levels [26, 32–34], and/or (iii) through

suppression of non-circulating unique NK cell subpopulations [44–46] of different susceptibility to stress hormones.

The current study also indicates that the duration of exposure of NK cells to stress hormones is a critical factor with potential confounding or misleading impact. The NK suppressive effects of cortisol increased over time (from 0 to 2 to 6 h of pre-incubation before being challenged with tumor cells), whereas the effects of PGE2 occurred immediately (0 h) and remained stable at different durations of pre-incubation with this hormone (i.e., at 2 and 6 h). These findings correspond well with the known nuclear transcriptional mechanisms activated by cortisol (and thus its delayed outcome) [41], and the immediate cytoplasmatic cAMP-dependent mechanisms activated by PGE2 [42] and epinephrine [3]. Therefore, when employing stress paradigms or *in vitro* exposure to stress hormones, the duration of the paradigm/exposure period may determine the impact of specific stress hormones, and such studies should thus include a complete time course for each hormone.

Clearly supporting the above conclusions are animal and human studies employing physiological levels of corticosterone or cortisol. *In vitro* studies, in which plasma was replaced by an artificial medium, demonstrated marked suppression of NKCC at physiological levels [18, 20]. However, a recent animal *in vivo* study demonstrated that no such suppression occurs within the animals at short exposure to corticosterone (less than 3 hr), and only a moderate suppression of NKCC appears following prolonged exposure (3–8 hr) to high physiological levels – an effect that was mainly ascribed to corticosterone-induced potentiation of an *in vivo* NK-suppressive effects of epinephrine [16]. A pivotal study in healthy humans also concluded that *in vivo* physiological changes in plasma cortisol levels along a one-hr period do not affect NKCC. Specifically, the administration of ACTH or metyrapone, which markedly increased or decreased plasma cortisol levels in these subjects, respectively, did not affect NK cytotoxicity levels. Additionally, no significant correlations were evident between levels of cortisol and levels of NK cytotoxicity, at baseline or following these manipulations, despite marked variations in both indices [21]. One factor that might explain the protective effects of plasma and of the *in vivo* milieu against suppression of NKCC by glucocorticoids is the presence of corticosteroid-binding-globulins, which bind up to 90% of glucocorticoid molecules [47], reducing the effective concentrations of glucocorticoids, and potentially buffering their effects [16].

Most importantly, our findings suggest that direct *in vivo* suppressive effects of epinephrine and PGE2 on NKCC would not be reflected in *ex-vivo* studies, while effects of glucocorticoids may be exaggerated. We demonstrated that when hormones are removed from the assay milieu, simulating their exclusion in the *ex-vivo* approach, the effects of epinephrine and PGE2 immediately vanish, while the effects of cortisol remain stable or increase with time. Thus, the interpretation of findings from numerous *ex-vivo* studies of NKCC, including our own, should be carefully reexamined, as is further elaborated below.

The current findings are aligned with recent animal studies we have conducted, directly indicating that an *in vivo* suppression of NKCC by PGE2 was not evident in an *ex-vivo* assessment, while a weaker *in vivo* effect of corticosterone was robustly evident *ex-vivo* [5], as would indeed be expected from the current study. Additionally, in rats' blood, *in vitro*

suppression of NKCC by PGE2 and by epinephrine vanished following their removal from the *in vitro* milieu, and even reversed (rebounded), while the impact of corticosterone persisted or increased after its removal [5], as similarly indicated herein in human blood.

To overcome some of the above limitations of the *ex-vivo* approach in humans, we have used a whole blood cytotoxicity assay, where NKCC is tested immediately upon blood withdrawal and without removing the plasma, its many factors, and the various cellular blood components [48–53]. We believe that compared to prolonged and manipulative procedures that enrich NK cells and eliminate the natural plasma milieu, this immediate whole blood approach better reflects the *in vivo* capacity of circulating NK cells to lyse target cells, as well as the modulation of this capacity by various stress responses.

As are most *in vitro* studies, the present study is clearly limited in simulating the complex *in vivo* milieu. Although hormonal concentrations in this study cover physiological levels, it is difficult to compare *in vitro* to *in vivo* concentrations, and local *in vivo* hormonal concentrations might be higher than their systemic levels, especially regarding catecholamines and PGs. Additionally, although plasma was maintained at some of the experimental conditions, one cannot assume the maintenance of all its factors and the simulation of their *in vivo* dynamics. Nevertheless, the current findings clearly suggest specific methodological impediments and distortions that are inherent to standard *in vitro* and *ex-vivo* approaches, and these same impediments have also been indicated in *in vivo* animal studies that do not suffer from most of the above limitations [5, 16].

It is worthy to note that the *in vitro* and *ex-vivo* approaches are valuable tools when their limitations are understood and circumvented. Specifically, the study of cellular and intracellular mechanisms is greatly advanced through *in vitro* approaches, albeit in an artificial context and commonly irrespective of significant modulating interactions with serum factors and other leukocytes [54–59]. The *ex-vivo* approach is unique in reflecting organ-specific cell redistribution (NK and others), and some, but not other, characteristics of these cell populations.

Additional prevalent obstacles to understanding the *in vivo* status of the NK cell system by *ex-vivo* and *in vivo* studies, which are not addressed herein, are (i) unrecognized stress-induced alterations in the number of NK cells in a cell population (*ex-vivo*) or in an immune compartment (*in vivo*) tested for NKCC, and (ii) the existence of NK cell subpopulations of different cytotoxic capacity, but of similar NK cell identifying markers [22, 44], that are differentially redistributed by stress hormones (also see Scheme 1). Such processes may lead to false interpretations, allegedly suggesting alterations in cytotoxicity per NK cell, while in fact the outcomes reflect NK cell trafficking or NK-subtype specific redistribution patterns. For example, unrecognized reduced proportion of NK cells (or of activated NK cells) within a sample of mononuclear cells tested for NKCC will result in lowered NKCC in such a sample, and will erroneously be interpreted as indicating suppression of NKCC per NK cell, while it might actually only reflect the reduction in the total number of NK cells (or activated NK cells) in the tested sample. Similarly, an *in vivo* reduction in NK cell number within the lungs' capillaries, where circulating tumors cells are being trapped and destroyed by marginating pulmonary NK cells [22, 44], may impact an *in vivo* index of NK activity

(e.g., lung tumor cell retention), not necessarily due to suppression of NKCC per NK cell. Thus, redistribution of NK cells, leading to reduction or enhancement in their numbers at specific strategic locations (e.g., lungs' capillaries or the skin) should be considered a regional immune suppression/enhancement. Such regional immune modulations are biologically significant [44, 60–62], and may bear clinical implications in cancer patients.

Although the direct effects of PGs and epinephrine on NKCC are not expected to be evident in *ex-vivo* assessments, some studies have reported *ex-vivo* enhancement of NKCC through these factors and following stress exposure [8, 9], and others reported suppression of NKCC [12–14, 17, 45, 63]. Therefore, we suggest that all these findings should be reinterpreted in light of insights from the present and other studies [5, 16]. *Ex-vivo* studies that reported enhanced NKCC by catecholamines may have reflected (i) a rebound effect induced by the removal of these hormones from the sample tested for cytotoxicity [5], (ii) an indirect effect of such hormones, and/or (iii) alterations in NK cell numbers or influx of more active NK cell subpopulation into the immune compartment studied. On the other hand, we reported *ex-vivo* suppression of NKCC by surgery, which was blocked by antagonizing catecholamines and PGs [22, 45, 64]. However, the blockade of catecholamines and PGs also reduced corticosterone levels [43, 64], which we now believe was the actual factor causing the evident *ex-vivo* suppression of NKCC and its attenuation by the antagonists to catecholamines and PGs. Irrespective of this *ex-vivo* suppression by corticosterone, we have found that the *in vivo* release of catecholamines and PGs following stress or surgery causes marked *in vivo* suppression of NKCC [12, 22, 31], without exerting these effects in *ex-vivo* assessments, and not through elevating corticosterone levels [5].

The finding presented herein should stimulate researchers to assess whether similar processes and fallacies exist with respect to the impact of stress on other immune indices. For example, cytokine levels may differentially be affected *in vivo* and *in vitro*, as is reported by a recent study regarding IL-12 regulation by stress hormones [43]. Antibody production seems to be a systemic prolonged process that may be less affected by transient stress responses. However, it may involve critical sub-processes, such as initial interactions with pathogens, which may be restricted to a specific immune compartment that is affected by leukocyte trafficking and specific regional humoral milieu. Clearly, each immune index should be studied in accordance with its unique characteristics.

In summary, the limitations of the *in vitro* and *ex-vivo* approaches studied herein stem from the inadequate representation and preservation of the natural *in vivo* milieu. Given the complex and dynamic nature of NK cell modulation by stress hormones and cytokines, also evident in the current study, these inadequacies are doomed to yield misleading outcomes, as specified above. In human studies, some distortions might be minimized by using a non-delayed whole blood NK cytotoxicity assay [53]. Understanding the specific limitations of *ex-vivo* and *in vitro* approaches in the context of NKCC, combining them with *in vivo* studies when feasible, and considering the potential impact of redistribution of subpopulations of NK cells by stress hormones, may help reinterpret previous literature and devise more advance future studies. Such considerations may also be applicable to other immune indices, especially those of a highly dynamic nature.

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The following are authors' definitions

In vitro: "in glass" (Latin). Studies in which the experimental manipulations and the assessment of the outcomes are both conducted in test-tubes, petri dishes, and alike. For example, studying the impact of epinephrine on cytotoxicity of purified NK cells - all conducted in testing tubes.

Ex-vivo: "out of the living" (Latin). Studies in which the experimental manipulations are conducted in the living organism, followed by *in vitro* assessments of the measured outcomes. For example, subjecting animals to swim-stress, followed by blood withdrawal and *in vitro* assessment of NK cytotoxicity.

In vivo: "within the living" (Latin). Studies in which the experimental manipulations are conducted in the living organism, followed by either (i) assessments of outcome indices in the living organism (e.g., imaging of a developing malignant mass or freezing behavior), or (ii) assessment of an index in an approach that undoubtedly preserves and reflects its status in the living organism (e.g., number of lung metastases counted following animal euthanasia, or serum antibody levels).

Highlights

- Exclusion of plasma renders NK cells 10–30 fold more susceptible to stress hormones
- *In vitro* (no plasma), physiological levels of CORT, PGE, and Epi suppress NKCC
- Acute physiological CORT levels are unlikely to affect NKCC *in vivo* (plasma milieu)
- Prolonged CORT exposure may suppress NKCC *in vivo;* effects may be inflated *ex-vivo*
- *In vivo* profound suppression of NKCC by PGE or Epi would not be detected *ex-vivo*

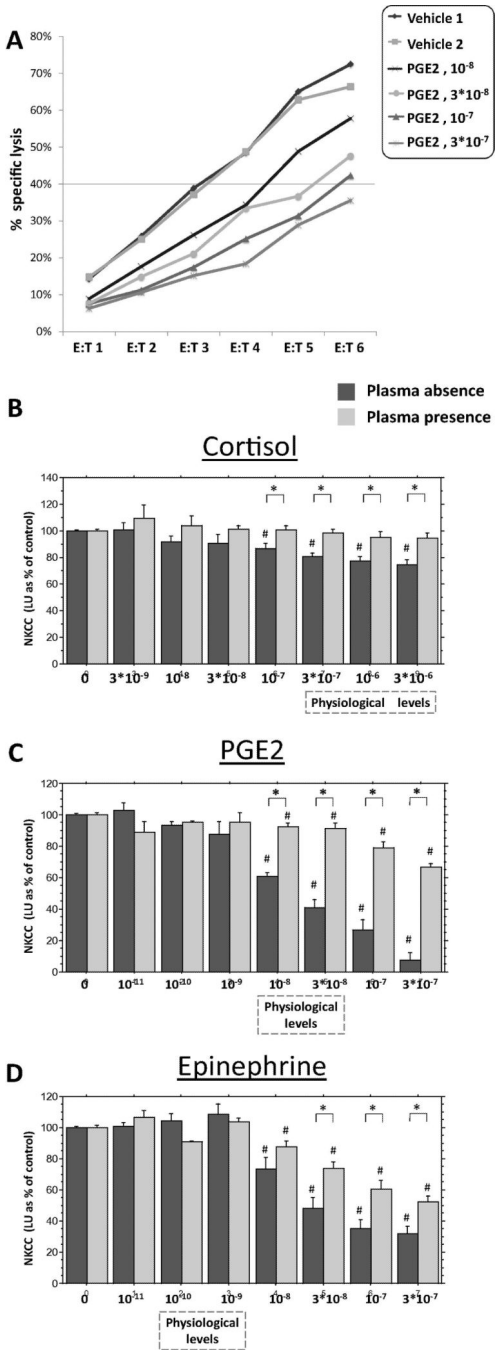


Fig. 1.

The effects of different hormones on NKCC against K562 target cells, in the presence of plasma and in its absence. (A) A representative E:T cytotoxicity curves in a single plate, including two replicates of control condition and 4 concentrations of PGE2, in the absence of plasma. Each curve is expressed as a single value (LU_{45}, as explained in details in the Method section), and all results are presented as % of control levels of this index (1B–D). (B) Cortisol dose-dependently suppressed NKCC, but only in the absence of plasma. (C) PGE2 and (D) epinephrine dose-dependently suppressed NKCC, but this suppression was

markedly more profound in the absence of plasma. ? Indicates a significant difference between the presence and absence of plasma. # Indicates a significant reduction in NKCC compared to the respective control (Vehicle) levels. The concentrations of epinephrine and PGE2 spanned beyond systemic physiological levels, as these hormones, but not cortisol, also interact locally with NK cells at higher concentrations. Data presented as mean + SEM.

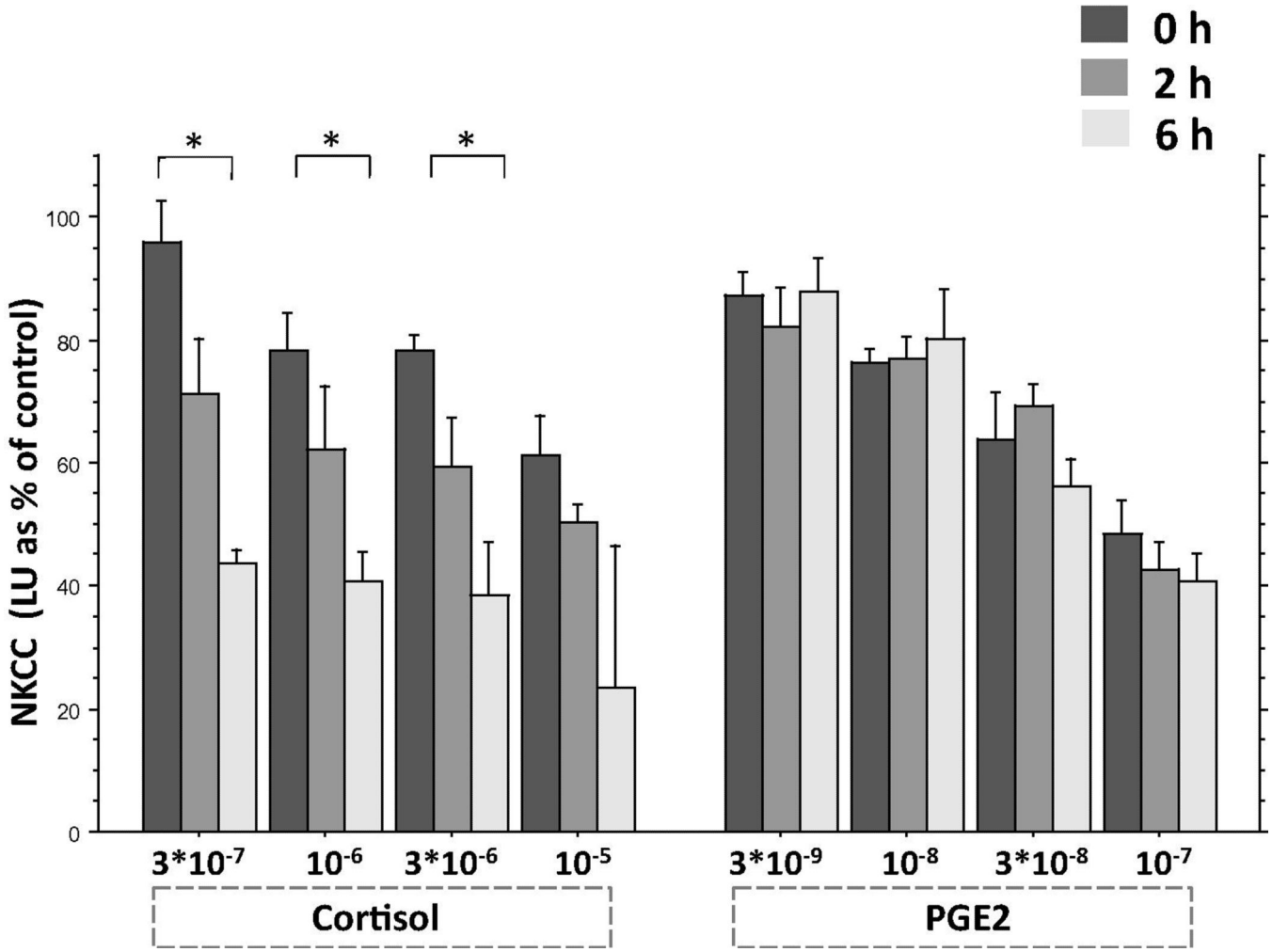


Fig. 2.

Exposure-time-dependent effects of cortisol and PGE2 on NKCC. Cortisol significantly reduced NKCC in 6 h more than in 0 h (indicated by *), whereas PGE2 exerts the same levels of suppression at all time-intervals. The figure presents the findings in the absence of plasma. Results are presented (mean + SEM) as % of vehicle (control condition) levels (not shown).

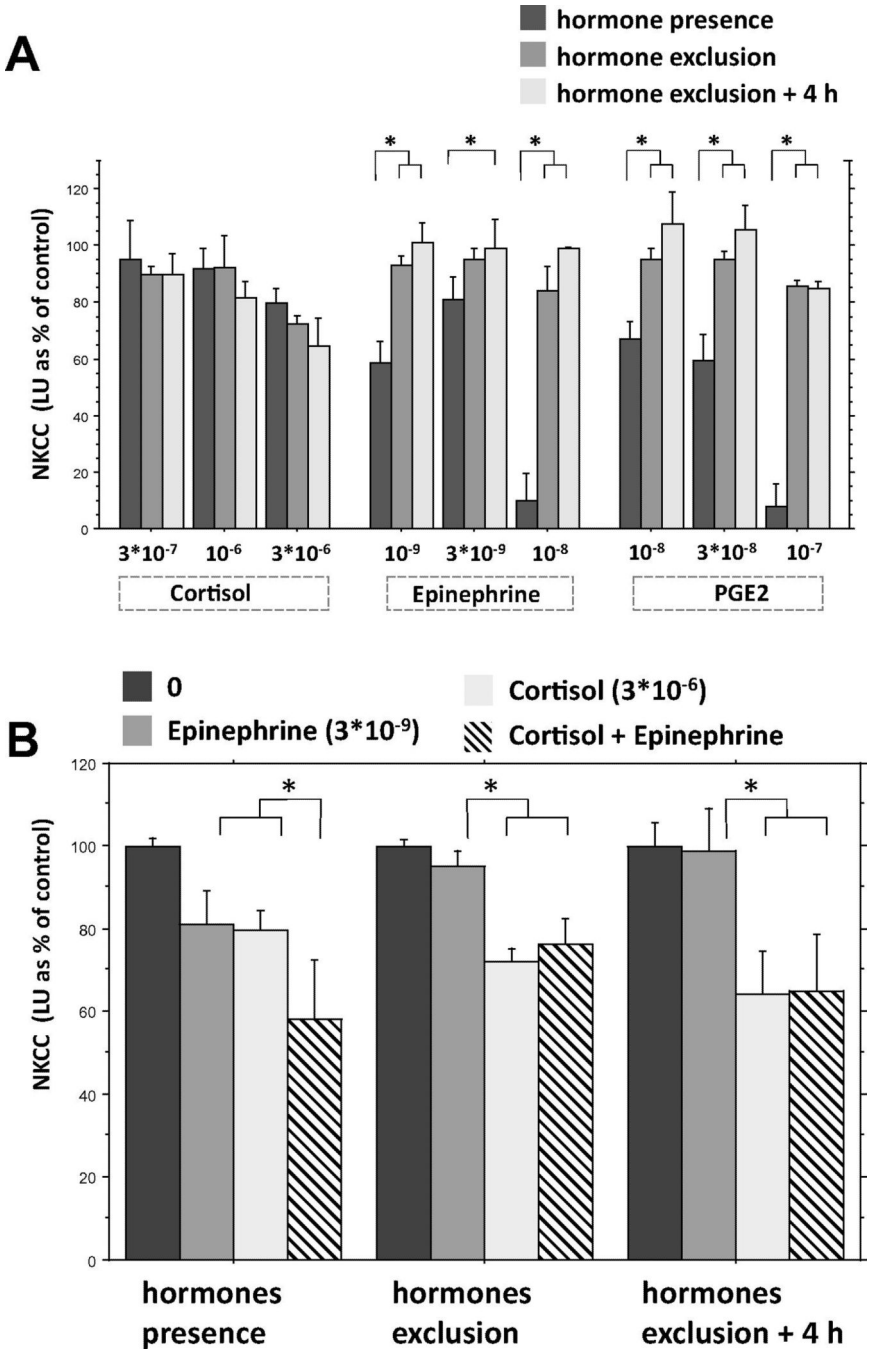
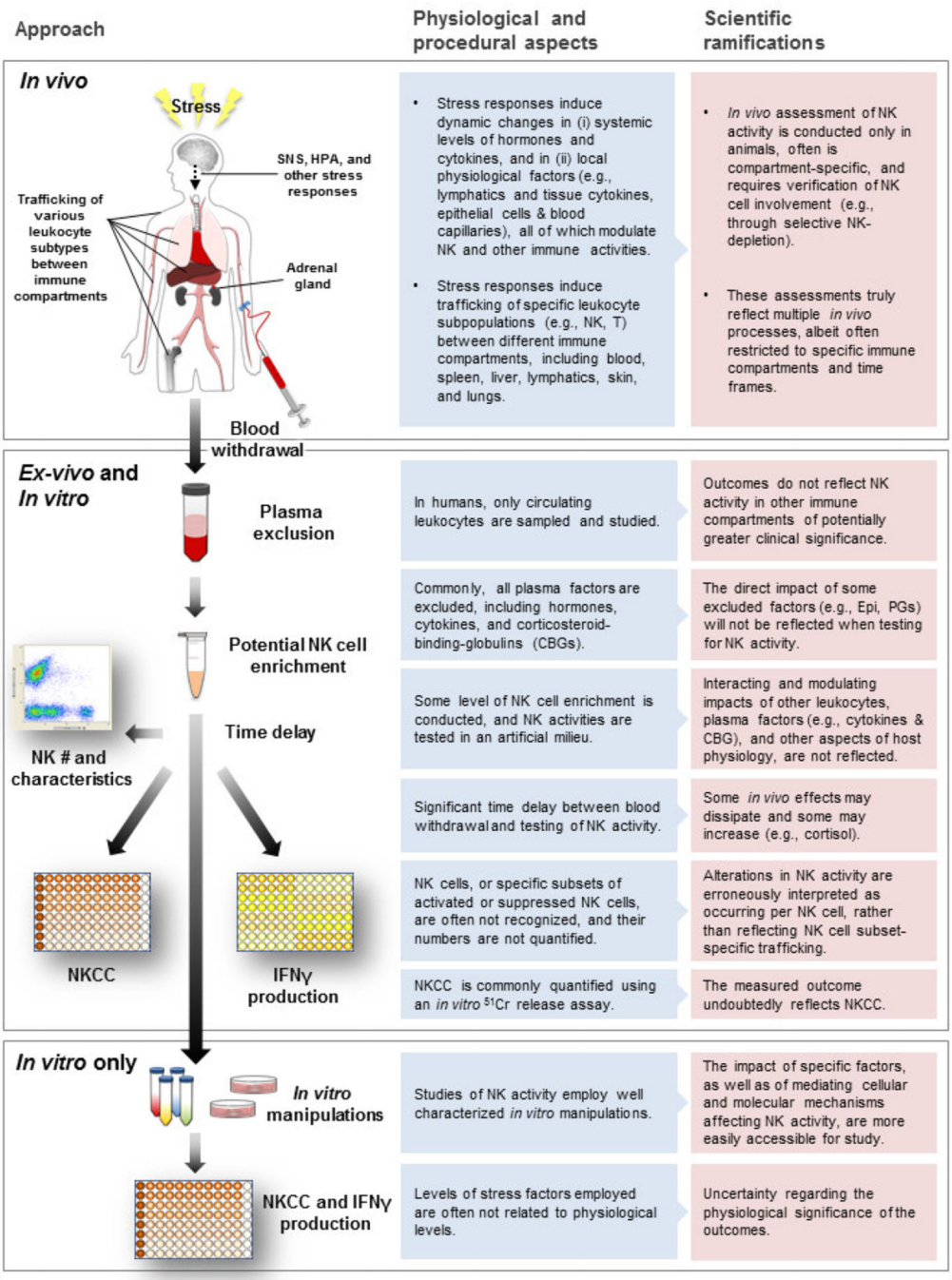


Fig.3.

The effects of different hormones on NKCC against K562 target cells. Hormones were either (i) maintained in the assay milieu while testing for NKCC (hormone presence), (ii) washed Jaway and blood immediately tested for of NKCC (hormone exclusion), or (iii) washed away and blood maintained at room temperature for 4 h before testing for NKCC (hormone exclusions + 4 h). (A) Epinephrine and PGE2 suppressed NKCC only when present in the *in vitro* setting, and their effects disappeared immediately following their removal, whereas suppression levels induced by cortisol did not change following its

removal. (B) In the presence of these hormones, the combination of cortisol and epinephrine yielded significantly greater suppression of NKCC than each of these hormones individually. Following the exclusion of the hormones and 4 hours later, the combination of cortisol and epinephrine suppressed NKCC similarly to cortisol alone, and significantly greater than epinephrine alone, for which the effects vanished. * Indicates a significant difference in NKCC levels between the different hormone impact approaches (A) or different drug combinations (B). Results are presented (mean + SEM) as % of vehicle (control condition) levels (not shown).



Scheme 1.

Different approaches to study the impact of stress on NK activity: Physiological and procedural aspects of the approaches and their scientific ramifications