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Regulation of human tissue kallikrein-related peptidase expression by steroid hormones in 32 cell lines

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Abstract

Human tissue kallikrein-related peptidases (KLK), which are secreted serine proteases, are encoded by 15 genes located on chromosome 19q13.4. Previous studies have shown that *KLK* expression is regulated by steroid hormones and many KLKs are dysregulated in hormone-dependent malignancies. Some KLKs are proposed biomarkers for these cancers. We have characterized KLK hormonal regulation patterns using a large number of human cell lines. KLK levels were quantified in supernatants from 32 cell lines, each subjected to four hormonal stimulations (dexamethasone, norgestrel, dihydrotestosterone or estradiol), using ELISAs. Cell lines included breast, prostate, ovarian, lung, pancreatic, colon, and cervical cancer cells, T-lymphocytes, keratinocytes and a non-cancerous epithelial breast cell line. KLKs were regulated in several cell lines not previously studied, such as keratinocytes (KLK5, 6, and 7), ovarian cancer (KLK5 and 9) and cervical cancer (KLK3, 5, 6, 7, 8, 10, 11, and 13) cells. Many KLKs were regulated by the synthetic glucocorticoid dexamethasone; specifically, KLK5, 6, 8, 10 and 11 were upregulated in several breast cancer cell lines and downregulated in several cervical cancer cell lines. Knowledge of KLK hormonal regulation patterns will help to shed further light on their potential use as biomarkers and therapeutic targets for hormone-related malignancies.

Keywords: cancer biomarkers; cell lines; gene regulation; kallikrein-related peptidases; serine proteases; steroid hormones.

Introduction

The human tissue kallikrein-related peptidase (KLK) family includes 15 genes located in tandem on chromosome 19q13.4 (Yousef and Diamandis, 2001). These genes encode secreted serine proteases with either chymotryptic- or tryptic-like activity (Yousef and Diamandis, 2001). The physiological functions of the 'classical kallikreins', KLK1, 2 and 3, are fairly well established; however the functions of the remaining 12 members of this family remain largely unknown. Recent studies have suggested potential roles for KLK6 in the central nervous system (Scarisbrick et al., 2006b) and in tissue remodeling by cleavage of extracellular matrix proteins (Bernett et al., 2002; Blaber et al., 2002; Ghosh et al., 2004; Kapadia et al., 2004; Michael et al., 2005; Rajapakse et al., 2005; Veveris-Lowe et al., 2005; Obiezu et al., 2006). Some KLKs are known to participate in proteolytic cascades, such as KLK5, 7 and 14 in skin (Caubet et al., 2004; Borgono et al., 2007) and KLK2, 3 and 5 in seminal plasma (Michael et al., 2006).

All *KLK*s have been shown to be under some form of steroid hormone regulation at the mRNA and protein levels in cancer cell lines (Borgono et al., 2004; Paliouras and Diamandis, 2007). Many KLKs are dysregulated in hormone-dependent malignancies such as breast, ovarian and prostate cancer (Borgono and Diamandis, 2004). Some KLKs are known, or candidate, biomarkers for these hormonally dependent cancers. For example, KLK3 (prostate-specific antigen or PSA) is a well-known biomarker for the diagnosis and monitoring of prostate cancer (Stamey et al., 1987), and KLK6 and 10 are candidate biomarkers for ovarian cancer (Diamandis et al., 2000; Luo et al., 2003).

Until now, KLK hormonal regulation has been investigated for individual KLKs and in very few cell lines, mostly at the mRNA level. Here, we use highly sensitive and specific ELISAs for each KLK to analyze the hormonal regulation patterns of 14 KLKs in 32 cell lines. Cells were stimulated with the synthetic glucocorticoid dexamethasone, the synthetic androgenic progestin norgestrel, the androgen dihydrotestosterone (DHT), and the estrogen 17β-estradiol. Cell lines included breast, ovarian, prostate, pancreatic, colon and cervical cancer cell lines, as well as a T-lymphocyte line, a keratinocyte line and a near-normal breast epithelial cell line. This experimental design allowed us not only to establish regulation patterns of each KLK by steroids, but also to examine parallel (coordinated) expression of KLKs in the different contexts of the 32 cell lines.

Results

Thirty-two cell lines were analyzed in triplicate for constitutive KLK expression levels (stimulation with vehicle alone) and after stimulation with four different steroid hormones: dexamethasone, norgestrel, DHT and estradiol. All cell lines used, their tissue of origin, response to hormone treatment, and hormone receptor status are shown in Table 1.

Constitutive expression without hormonal regulation

The following cancer cell lines constitutively expressed at least one KLK but showed no significant change in KLK concentration after stimulation by any of the four

steroid hormones: A-427 (lung), HTB-177 (lung), BT-20 (breast), MDA-231 (breast), PC3 (prostate), DU-145 (prostate), Jurkat (T-lymphocytes), ES-2 (ovarian), OvCar-5 (ovarian), TOV-112D (ovarian), TOV-21G (ovarian), MIA-PaCa (pancreatic), HeLa (cervical), C-33A (cervical), C-4i (cervical) and Colo-320 (colon). Detailed data are shown in Table 2. Some of these cell lines expressed relatively very high levels (>10 μg/l) of some KLKs. KLK1 was not detected in any of the cell lines analyzed.

Constitutive expression with hormonal regulation

In Table 3 we present quantitative data on KLK expression before and after stimulation of cell lines with each

Table 1 List of cell lines used in the study along with their tissue of origin, response to hormone treatment and hormone receptor status.

Cell line	Tissue of origin	Hormone response	Receptor status	References
BT-20	Breast	Negative	Negative	Engel and Young, 1978
MCF-7	Breast	DHT	AR+	Hall et al., 1990
		Norgestrel	PR+	Hall et al., 1990
		Estrogen	ER+	Hall et al., 1990
		Dexamethasone	GR+	Onda et al., 2006
MDAMB-231	Breast	Negative	Negative	Engel and Young, 1978
MDA-MB-468	Breast	Dexamethasone	GR+	Kulasingam and Diamandis, 2007
T-47D	Breast	DHT	AR+	Hall et al., 1990
		Norgestrel	PR+	Hall et al., 1990
		Estrogen	ER+	Hall et al., 1990
BT-474	Breast	DHT	AR+	Hall et al., 1990
		Norgestrel	PR+	Hall et al., 1990
MCF-10A	Breast	Estrogen	ER-	Pilat et al., 1996
		Dexamethasone	GR+	Kulasingam and Diamandis, 2007
ES-2	Ovarian	Negative	Negative (ER)	Ding et al., 2006
CaOv-3	Ovarian	DHT	Unknown	
		Estrogen	ER+	Jones et al., 1994
		Dexamethasone	Unknown	
Ov-90	Ovarian	Estrogen	Unknown	
OvCar-5	Ovarian	Negative	Unknown	
TOV-112D	Ovarian	Negative	Unknown	
TOV-21G	Ovarian	Negative	Unknown	
LNCaP	Prostate	Norgestrel	PR+	Sasaki et al., 2002
		Estrogen	ER+	Sasaki et al., 2002
DU-145	Prostate	Negative	ER-, AR+, PR+	Sasaki et al., 2002 Castagnetta et al., 1990
PC3	Prostate	Negative	ERβ+, AR-, PR+	Sasaki et al., 2002 Castagnetta et al., 1990
$PC3(AR)_6$	Prostate	DHT	AR+	Transfected
		Norgestrel	PR-	Castagnetta et al., 1990
22-RV1	Prostate	DHT	AR+	Hartel et al., 2004
		Norgestrel	PR+	Hartel et al., 2004
		Estrogen	ER-	Hartel et al., 2004
Ht-3	Cervical	Dexamethasone	Unknown	
Caski	Cervical	Estrogen	ER+	Macinga et al., 1995
		Dexamethasone	Unknown	
C-33A	Cervical	Negative	AR+	Sager et al., 2003
C-4i	Cervical	Negative	negative	Sager et al., 2003
SiHa	Cervical	Negative	ER+, PR-	Ruutu et al., 2006
Ms751	Cervical	Norgestrel	Unknown	
		Estrogen	Unknown	
		Dexamethasone	Unknown	
HeLa	Cervical	Negative	ER-, PR+	Somasekhar and Gorski, 1988
Me-180	Cervical	DHT	AR+	Sager et al., 2003
		Estrogen	ER+	Macinga et al., 1995
		Dexamethasone	GR+	Sager et al., 2003
Colo-320	Colon	Negative	Unknown	
MIA-Paca	Pancreatic	Negative	AR+	Corbishley et al., 1986
HaCat	Keratinocyte	Estrogen	ER-	Planas-Silva et al., 1999
		Dexamethasone	GR+	Onda et al., 2006
Jurkat	T-lymphocyte	Negative	Negative	Berman et al., 1991, Bamberger et al., 1997
A-427	Lung	Negative	Unknown	
HTB-177	Lung	Negative	Unknown	

of four steroid hormones. Representative RT-PCR data for the stimulation of two cell lines (Ms-751 and MCF-7) are shown in Figure 1. Clearly, the KLK most frequently constitutively expressed at relatively high levels (>10 μg/l) and in diverse cell lines is KLK5, followed by KLK9, 10, 11, 6 and 8. In addition, KLK2 and 3 were frequently highly expressed in prostate cancer cell lines. Based on the data in Table 3, we classified KLK concentration changes after hormone stimulation into three categories: (a) dramatic, >500-fold change from baseline; (b) pronounced, at least 5-fold but less than 500-fold change from baseline; and (c) moderate, between 2- and 5-fold change from baseline. These data are summarized in Table 4. Changes in KLK concentration that were statistically significant but less than 2-fold are listed in Table 3, but these changes are considered small and are not discussed further. In cases for which the baseline KLK concentration was below the detection limit of the assay, the detection limit was used for calculating the fold change. Based on the data in Table 4, the following conclusions can be drawn (summarized in Table 5):

Table 2 Cell lines constitutively expressing KLK but without changes after stimulation by steroid hormones.

Cell line	Cancer type	KLK	Concentration, mean±SD (μg/l)
BT-20	Breast	7	0.2±0.09
		8	1.9±0.4
		9	**13±4**
		15	0.25±0.06
MDA-231	Breast	7	0.4±0.08
		9	**36±2**
		15	0.3±0.03
PC3	Prostate	5	0.5±0.3
		6	0.8±0.2
		9	7.5±1
DU-145	Prostate	9	8.8±5.4
OvCar-5	Ovarian	6	4.6±0.37
		9	**15±2.2**
		10	2.6±1.5
		11	4.6±0.2
ES-2	Ovarian	9	**13.4±5.5**
TOV-112D	Ovarian	7	0.7±0.04
		9	1.2±0.5
TOV-21G	Ovarian	8	0.45±0.0006
C-4i	Cervical	5	**65±2.1**
		6	1.1±0.1
		7	1.1±0.1
		8	2.9±0.2
		9	3.1±0.68
		10	**48±14**
C-33A	Cervical	7	0.35±0.05
		9	0.35±0.1
		15	0.23±0.04
HeLa	Cervical	7	0.3±0.17
		9	5.0±2.9
Colo-320	Colon	2	0.008±0.002
		11	0.45±0.26
		13	0.47±0.19
MIA-PaCa	Pancreatic	5	**43±6.3**
		6	**18±1.3**
		7	3±0.15
		8	1.9±0.15
		9	2.9±0.5
		10	0.11±0.01
		13	0.2±0.04
A-427	Lung	9	**24±8**
		10	0.7±0.4
HTB-177	Lung	9	4±0.5
		10	0.2±0.1
		11	0.16±0.03
Jurkat	T-lymphocyte	9	1.9±0.4

Values of KLK expression above 10 μg/l are shown in bold.

- KLK2 and 3 were dramatically upregulated by androgens and progestins in breast cancer cell lines. Similarly, pronounced upregulation of KLK2 and 3 by androgens and progestins was observed in prostate cancer cell lines. KLK2 was also upregulated to a smaller degree by estrogens in the LNCaP prostate cancer cell line.
- KLK5 was upregulated by estrogens in breast cancer cell lines and downregulated by dexamethasone in cervical and breast cancer cell lines.
- KLK6 was upregulated by estrogens and dexamethasone in breast cancer cell lines, but downregulated by dexamethasone in cervical cancer cell lines.
- KLK8 was upregulated by estrogens and dexamethasone in breast cancer cell lines, but downregulated by dexamethasone in cervical cancer cell lines.
- KLK10 was upregulated by androgens, estrogens and dexamethasone in breast cancer cell lines, but downregulated by dexamethasone in the near-normal breast epithelial cell line MCF-10A.
- KLK11 was upregulated by estrogens, dexamethasone, androgens and progestins in breast cancer cell lines and by estrogens in cervical cancer cell lines, but was downregulated by dexamethasone in cervical cancer cell lines.
- KLK13 was downregulated by dexamethasone in cervical cancer cell lines.
- KLK14 was upregulated by estrogens in breast cancer cell lines.
- KLK15 was upregulated by estrogens in prostate cancer cell lines.

KLK9 was widely constitutively expressed in many of the cell lines analyzed (Table 2) but was not hormonally regulated in any of the cell lines. KLK7 was also expressed constitutively by many of the cell lines tested, but was only hormonally regulated in keratinocytes (HaCat) by dexamethasone (Table 3). We did not include KLK12, since the ELISA for this KLK is not yet fully validated. KLK1 was not detected in any of the 32 cell lines tested.

Many KLKs appear to be co-regulated by certain hormones in specific cell lines, such as KLK6, 10 and 11 upregulation by dexamethasone in MCF-7 cells (Figure 2A–C), downregulation of KLK5 and 6 by dexamethasone in Ms-751 cells (Figure 2D,E), downregulation of KLK5, 6, 8, 11 and 13 in Ht-3 cells (Figure 3) and upregulation of KLK6, 11 and 14 by estradiol in BT-474 cells (Figure 4).

Table 3 Hormonal regulation results for KLKs in specific cell lines.

Hormone	KLK	Cell line (type)	Level (μg/l)		Fold change
			Constitutive	Regulated	
Dexamethasone	5	MDA-MB-468 (breast cancer)	640±29	830±78‡	+1.4
		MCF-10A (normal breast)	0.25±0.04	0.06±0.03‡	-5
		CaOv-3 (ovarian cancer)	227±17	292±7.8‡	+1.3
		HaCat (keratinocytes)	88±28	107±9.5‡	+1.2
		Ms-751 (cervical cancer)	84±2	6‡	-14
		Me-180 (cervical cancer)	4.6±0.7	1.9±0.3‡	-2.4
		Ht-3 (cervical cancer)	235±25	133±6.5‡	-1.8
		Caski (cervical cancer)	96±3	83±3.2‡	-1.2
	6	MCF-7 (breast cancer)	0.3±0.07	2.2±0.3‡	+7.3
		Ms-751 (cervical cancer)	0.7±0.06	0.08±0.02‡	-8.8
		Ht-3 (cervical cancer)	9.9±0.6	4.4±0.36‡	-2.2
		Caski (cervical cancer)	1.3	1.1±0.06‡	-1.2
	7	HaCat (keratinocytes)	3.2±0.5	5.7±0.6‡	+1.8
	8	MDA-MB-468 (breast cancer)	2.1±0.06	4.1±1.1‡	+2.3
		Ms-751 (cervical cancer)	0.3±0.05	0.14±0.05†	-2.1
		Ht-3 (cervical cancer)	17±1.6	9.6±1.8†	-1.8
	10	MCF-7 (breast cancer)	<0.05	2.1±0.3‡	+42
		MDA-MB-468 (breast cancer)	21±3.5	38±6.7‡	+1.8
		MCF-10A (normal breast)	2.8±1.3	0.3±0.1†	-9
		Ht-3 (cervical cancer)	52±3.5	61‡	+1.2
	11	MCF-7 (breast cancer)	2.3±0.6	21±7‡	+9.1
		Me-180 (cervical cancer)	125±13	68±19‡	-1.8
		Ht-3 (cervical cancer)	4.2±0.26	1.8±0.45‡	-2.3
	13	Ht-3 (cervical cancer)	1.3±0.1	0.37±0.06‡	-3.5
DHT	2	22RV1 (prostate cancer)	5±2.5	49±21†	+9.8
	2	LnCaP (prostate cancer)	28.5±7.0	55±11	+1.9
	2	BT-474 (breast cancer)	<0.005	20±4.4‡	+4000
	2	T-47D (breast cancer)	<0.005	0.02±0.01	+4.0
	3	LnCaP (prostate cancer)	240±75	404±68	+1.7
	3	BT-474 (breast cancer)	<0.005	6.6±0.7‡	+1320
	3	T-47D (breast cancer)	<0.005	0.36±0.3	+72
	5	PC3(AR)6 (prostate cancer)	1372±133	2413±373†	+1.8
		CaOv-3 (ovarian cancer)	227±17	155±29‡	-1.5
	8	T-47D (breast cancer)	0.09±0.02	0.13±0.03	+1.4
	8	$PC3(AR)_6$ (prostate cancer)	2.5±0.5	5.8±0.36	+2.3
	10	T-47D (breast cancer)	0.7±0.04	8.3±0.27‡	+11.9
		MCF-7 (breast cancer)	<0.05	0.53±0.28†	+10.6
	11	Me-180 (cervical cancer)	125±13	73±30†	-1.7
	11	T-47D (breast cancer)	0.08±0.02	0.27±0.03†	+3.4
	11	MCF-7 (breast cancer)	2.3±0.6	9.4±2.9†	+4.1
Norgestrel	2	22RV1 (prostate cancer)	5±2.5	76±36‡	+15.2
	2	LNCaP (prostate cancer)	28±7	109±37‡	+3.9
	2	BT-474 (breast cancer)	<0.005	17.5±7.6‡	+3500
	2	T-47D (breast cancer)	<0.005	35±18†	+7000
	3	22RV1 (prostate cancer)	68±50	694±317‡	+10.2
	3	BT-474 (breast cancer	<0.005	2.9±1.8‡	+580
	3	T-47D (breast cancer)	<0.005	292±177‡	+58400
	5	Ms-751 (cervical cancer)	84±2	116±12.5†	+1.4
	8	T-47D (breast cancer)	0.09±0.02	0.12±0.007	+1.3
	8	$PC3(AR)_6$ (prostate cancer)	2.5±0.5	4.3±0.6	+1.7
	10	MCF-7 (breast cancer)	<0.05	0.13±0.03	+2.6
	11	MCF-7 (breast cancer)	2.3±0.6	8.8±2.8	+3.8
	15	LNCaP (prostate cancer)	0.8±0.3	1.1±0.4†	+1.4
Estradiol	2	22RV1 (prostate cancer)	5±2.5	39±4†	+7.8
	2	LNCaP (prostate cancer)	28±7	83.4±30.7†	+3.0
	3	LNCaP (prostate cancer)	297±111	639±143	+2.2
	5	MCF-10A (normal breast)	0.25±0.04	0.43±0.04‡	+1.7
		HaCat (keratinocytes)	88±28	137±19‡	+1.6
		Ms-751 (cervical cancer)	84±2	78±4†	-1.1
		Caski (cervical cancer)	96±3	112±2.9‡	+1.2
		MCF-7 (breast cancer)	<0.05	0.16±0.03‡	+3.2
	6	HaCat (keratinocytes)	1.6±0.1	2.1±0.3‡	+1.3
		Caski (cervical cancer)	1.3	1.5±0.1†	+1.2
		BT-474 (breast cancer)	<0.2	0.24±0.03‡	+1.2
		MCF-7 (breast cancer)	0.32±0.07	2.1±0.1‡	+6.6
	8	T-47D (breast cancer)	0.08±0.02	0.42±0.07†	+5.3

Table 3 (Continued)

Hormone	KLK	Cell line (type)	Level (μg/l)		Fold change
			Constitutive	Regulated	
	9	CaOv-3 (ovarian cancer)	3.2±0.3	5.7±0.98‡	+1.8
	10	MCF-7 (breast cancer)	<0.05	0.38±0.07	+7.6
	11	Me-180 (cervical cancer)	0.15±0.03	0.4±0.07‡	+2.7
		BT-474 (breast cancer)	2.3±0.58	12.6±5.7‡	+5.5
		MCF-7 (breast cancer)	0.11±0.02	2.8±0.6‡	+25.5
	14	BT-474 (breast cancer)	<0.05	0.17±0.03‡	+3.4
	15	LNCaP (prostate cancer)	0.8±0.3	1.6±0.6‡	+2.0
		Ov-90 (ovarian cancer)	0.4±0.1	0.38±0.2†	-1.1

Results are mean±SD. ‡$p<0.01$; †$p<0.05$.

Discussion

Several studies have previously demonstrated hormonal regulation of kallikrein-related peptidases in certain cancer cell lines. Cell lines were incubated for 7 days following hormonal stimulation to allow sufficient time for accumulation of KLK levels for analysis. This protocol has been optimized and we found no significant changes in cell death between stimulations.

Our study confirms and extends all previously published findings. We also report KLK hormonal regulation in many cell lines not previously studied (Table 3). In a few instances, changes in KLK expression upon hormonal stimulation were dramatic; we divided the changes in KLK expression into three groups: dramatic, pronounced and moderate (Table 4). These data allowed us to summarize the hormonal regulation of each KLK by various steroids, as shown in Table 5. We also included representative RT-PCR data from two cell lines, Ms-751 and MCF-7, in Figure 1 for comparison. The RT-PCR data match our protein expression data.

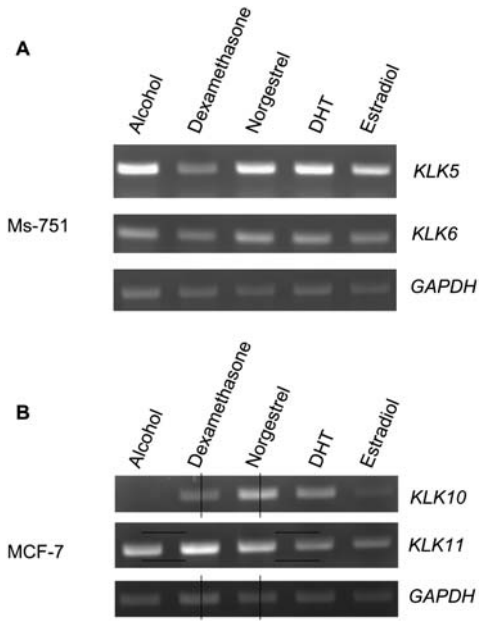

Figure 1 Ms-751 (A) and MCF-7 (B) cells were stimulated with either dexamethasone, norgestrel, DHT or estradiol (all 10^{-8} M final concentration) for 24 h.
Total RNA was collected and reverse-transcribed using first-strand synthesis. RT-PCR was performed using *KLK*-specific primers and observed by staining of agarose gels with ethidium bromide. *GAPDH* was used as a control for expression levels between stimulations.

In several of the cell lines examined, KLKs were not under any form of steroid hormone regulation, but were constitutively expressed (Table 2). Many of these findings were expected, given that many of these cell lines are known to be steroid receptor-negative (Table 1). We did not find KLK expression (with the exception of KLK9) or regulation in Jurkat cells, as previously demonstrated (Scarisbrick et al., 2006a), although the receptor status of these cells is equivocal.

Notably, we report relatively high levels of KLK5 and 6, and to a lesser degree KLK7, in the pancreatic cancer cell line MIA-PaCa (Table 2). This finding is of interest, given that it was previously demonstrated that KLK6 is expressed by pancreatic islets (Petraki et al., 2002) and it has been suggested that KLK7 increases the invasiveness of pancreatic cancer cells (Johnson et al., 2007). Our data suggest that some KLKs may play a role in pancreatic cancer.

It has previously been shown that many KLKs are regulated by progestins (Magklara et al., 2000; Kishi et al., 2003; Luo et al., 2003; Borgono and Diamandis, 2004; Paliouras and Diamandis, 2007), androgens (Magklara et al., 2000; Kishi et al., 2003; Luo et al., 2003; Paliouras and Diamandis, 2007) and/or estrogens (Henttu et al., 1992; Kishi et al., 2003; Luo et al., 2003; Borgono and Diamandis, 2004; Paliouras and Diamandis, 2007) in breast and prostate cancer cells. Here, we report for the first time that KLKs are regulated by steroid hormones in many cervical cancer cells, especially by dexamethasone (mainly downregulation; Table 5). In the majority of cell lines in which hormonal regulation occurred, the appropriate steroid hormone receptor is known to be expressed; for some cell lines the hormone receptor status is unknown (Table 1). Since the regulation of KLKs by estrogens, androgens and progestins has been reported and discussed previously, we concentrate on our novel findings on KLK regulation by dexamethasone.

We have shown that many KLKs are regulated by the synthetic glucocorticoid dexamethasone, in some cases quite dramatically. Our recent study (Kulasingam and Diamandis, 2007) suggested that KLK10 is differentially regulated by glucocorticoids (GCs) in the breast cancer cell line MCF-7 (upregulated) and the near-normal breast epithelial cell line MCF-10A (downregulated). Here, we expand on this finding and report that many KLKs, in addition to KLK10, are regulated by GCs in breast cancer

Table 4 Regulation of KLK expression levels by steroid hormones.

Hormone	Regulation		
	Dramatic (>500-fold)	Pronounced (>5-fold, <500-fold)	Moderate (2- to 5-fold)
Dexamethasone		KLK5 (↓Ms-751) KLK6 (↑MCF-7) KLK6 (↓Ms-751) KLK10 (↑MCF-7) KLK10 (↓MCF-10A) KLK11 (↑MCF-7)	KLK5 (↓MCF10A) KLK5 (↓Me-180) KLK6 (↓Ht-3) KLK8 (↑MDA-MB-468) KLK8 (↓Ms-751) KLK11 (↓Ht-3) KLK13 (↓Ht-3)
DHT	KLK2 (↑BT-474) KLK3 (↑BT-474)	KLK2 (↑22RV1) KLK10 (↑MCF-7) KLK10 (↑T-47D)	KLK10 (↑MCF-7) KLK11 (↑T-47D) KLK11 (↑ MCF-7)
Norgestrel	KLK2 (↑BT-474) KLK2 (↑T-47D) KLK3 (↑BT-474) KLK3 (↑T-47D)	KLK2 (↑22RV1) KLK3 (↑22RV1)	KLK11 (↑MCF-7)
Estradiol		KLK2 (↑22RV1) KLK6 (↑MCF-7) KLK10 (↑MCF-7) KLK11 (↑MCF-7) KLK11 (↑BT-474)	KLK2 (↑LNCaP) KLK5 (↑MCF-7) KLK8 (↑T-47D) KLK11 (↑Me-180) KLK14 (↑BT-474) KLK15 (↑LNCaP)

The fold change was calculated based on basal expression (no steroid hormone stimulation) as explained in materials and methods.

Table 5 Summary of KLK hormonal regulation (>2-fold) by steroids in different cell lines.

KLK	Upregulation (cell lines)	Downregulation (cell lines)
2	Androgens, progestins (breast; prostate) Estrogens (prostate)	– –
3	Androgens, progestins (breast; prostate)	–
5	Estrogens (breast)	Dexamethasone (cervical; breast)
6	Estrogens, dexamethasone (breast)	Dexamethasone (cervical)
8	Estrogens, dexamethasone (breast)	Dexamethasone (cervical)
10	Androgens, dexamethasone, estrogens (breast)	Dexamethasone (breast)
11	Estrogens (breast; cervical) Dexamethasone, androgens, progestins (breast)	Dexamethasone (cervical)
13	–	Dexamethasone (cervical)
14	Estrogens (breast)	–
15	Estrogens (prostate)	–

For KLKs not listed, we did not detect changes that were >2-fold than baseline after hormonal stimulation. Smaller but statistically significant changes are shown in Table 2.

(upregulated) and cervical cancer cells (downregulated) (Table 5). Such KLKs include KLK6, 8, 10, and 11 (upregulation) and KLK5, 6, 8, 10, 11 and 13 (downregulation).

GCs are stress-induced steroid hormones and are known to function in the metabolism of glucose, protein and fat (Schoneveld et al., 2004). These hormones are synthesized in the adrenal cortex and released into the bloodstream in response to stress (Duma et al., 2006). GCs interact with cytoplasmic glucocorticoid receptors (GRs), resulting in GR dissociation from chaperone proteins, dimerization and translocation into the nucleus (Lu and Cidlowski, 2006).

GRs are ubiquitously expressed and function to regulate gene transcription through their binding to glucocorticoid responsive elements (GREs) on the DNA of target genes (Lu and Cidlowski, 2006). This regulation of transcription can be positive or negative, depending on the type of GRE present. Some GREs cause repression of transcription and are called negative GREs (nGREs) (Schoneveld et al., 2004). GRs have also been shown to interact with so-called GRE half sites (Table 6) resulting in the activation of transcription (Schoneveld et al., 2004). GRs are also known to interact with various transcription factors such as activator protein (AP-1), signal transducers and activators of transcription (STATs) and nuclear factor (NF)-κB (Adcock, 2001; Lu and Cidlowski, 2006). These interactions provide another means by which GRs modify gene expression.

In this study KLK6, 8, 10 and 11 were upregulated by a GC in several breast cancer cell lines and KLK5, 6, 8, 10, 11 and 13 were downregulated by a GC in many cervical cancer cell lines and in the near-normal breast cell line MCF-10A (KLK5 and 10). For example, KLK6, 10 and 11 were highly upregulated in MCF-7 (breast cancer) cells (Figure 2A–C), whereas KLK5 and 6 were highly downregulated in Ms-751 (cervical cancer) cells (Figure

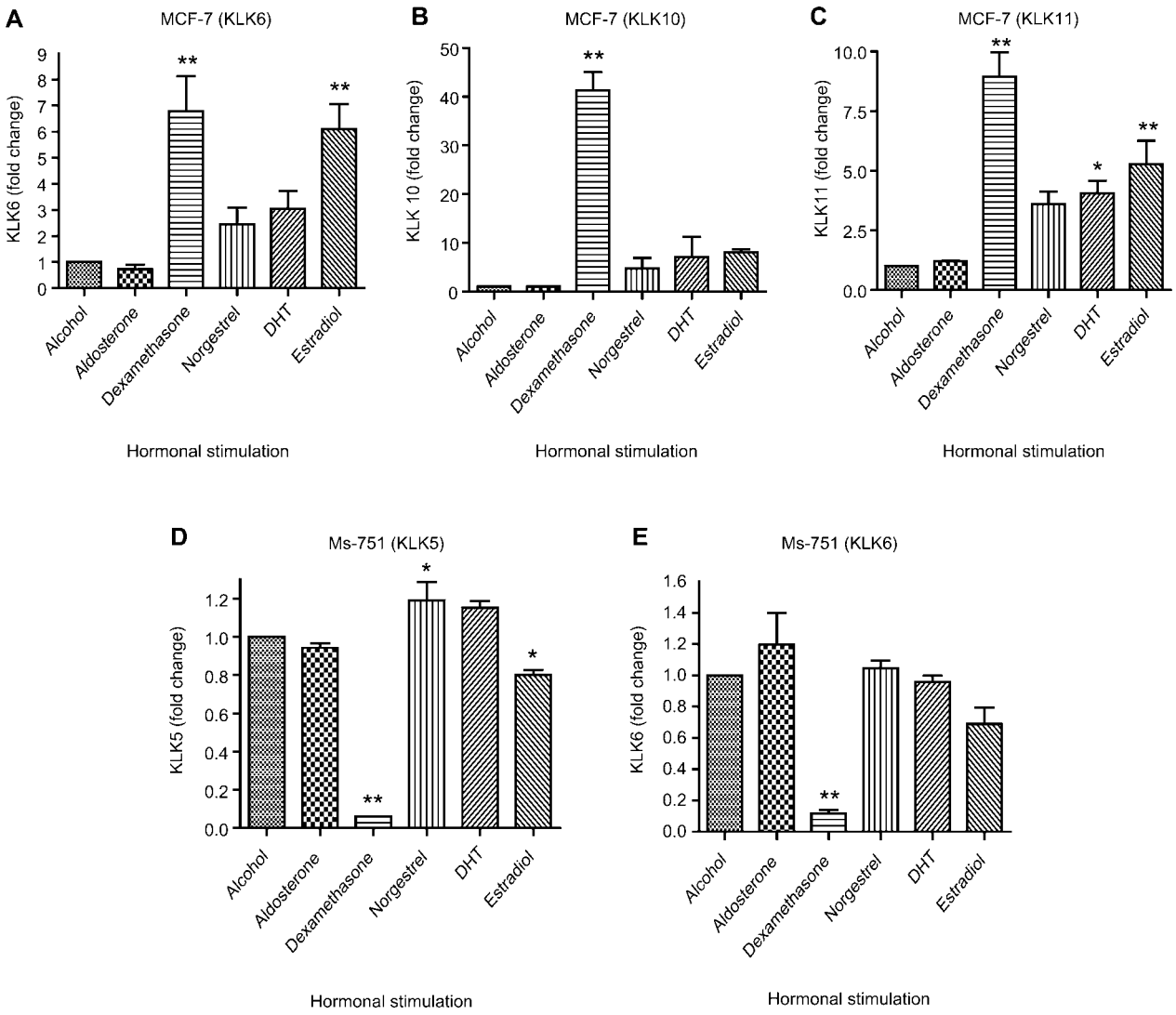

Figure 2 Fold changes in KLK 6 (A), 10 (B) and 11 (C) expression in MCF-7 breast cancer cells and KLK 5 (D) and 6 (E) expression in Ms-751 cervical cancer cells upon stimulation with dexamethasone, norgestrel, DHT or estradiol.
Alcohol was the solvent (negative control). Fold changes were calculated based on the basal level of expression upon alcohol treatment. $^{**}p<0.01$; $^{*}p<0.05$.

2D,E) in response to dexamethasone. The upregulation of KLK10 in MCF-7 and its downregulation in MCF-10A cells by dexamethasone have previously been reported (Kulasingam and Diamandis, 2007). Similar downregulation of KLK5 in MCF-10A was observed in the present study (Table 4).

Recently, we performed high-resolution cytogenetic analysis of the KLK locus in various breast and ovarian cell lines and found frequent unbalanced translocations. In particular, the breast cancer cell line MCF-7 harbors unbalanced translocations of the KLK locus, whereas the cell line MCF-10A does not (our unpublished data). It is possible that dexamethasone downregulates KLKs in normal breast (and cervical) cells, but upregulates KLKs in cells in which the KLK locus (and its presumed regulatory elements) are translocated to other genomic regions. This speculation needs experimental verification.

Groups of KLKs appear to be co-regulated by dexamethasone in cervical cancer cell lines. One striking example for the cervical cancer cell line Ht-3 is the co-downregulation of KLK5, 6, 8, 11, and 13 (Figure 3). In Figure 2 we show the co-upregulation of KLK5 (not shown), 6, 10 and 11 by estradiol in MCF-7 cells and in Figure 4 we further show the co-upregulation of KLK6, 11 and 14 by estradiol in BT-474 cells. In MCF-7 cells, dexamethasone co-upregulated KLK6, 10 and 11 (Figure 2).

Previously, we speculated that KLKs act within highly regulated proteolytic cascades (Borgono and Diamandis, 2004). The KLK co-regulation patterns identified here and elsewhere (Paliouras and Diamandis, 2007) (Table 5 and Figures 2–4) support this hypothesis.

As previously mentioned, GRs activate or repress transcription through their binding to GREs within the promoter region of a target gene (Lu and Cidlowski, 2006). We used bioinformatics to analyze the genomic regions 3.0 kb upstream of *KLK5, 6, 7, 8, 10, 11* and *13* genes for the presence of GREs, nGREs and GRE half sites (Table 6). We identified putative GRE half sites (Schoneveld et al., 2004) upstream of *KLK5, 6, 7, 8, 10, 11* and *13* genes. The presence of these sites may explain the ability of dexamethasone to modulate KLK expression.

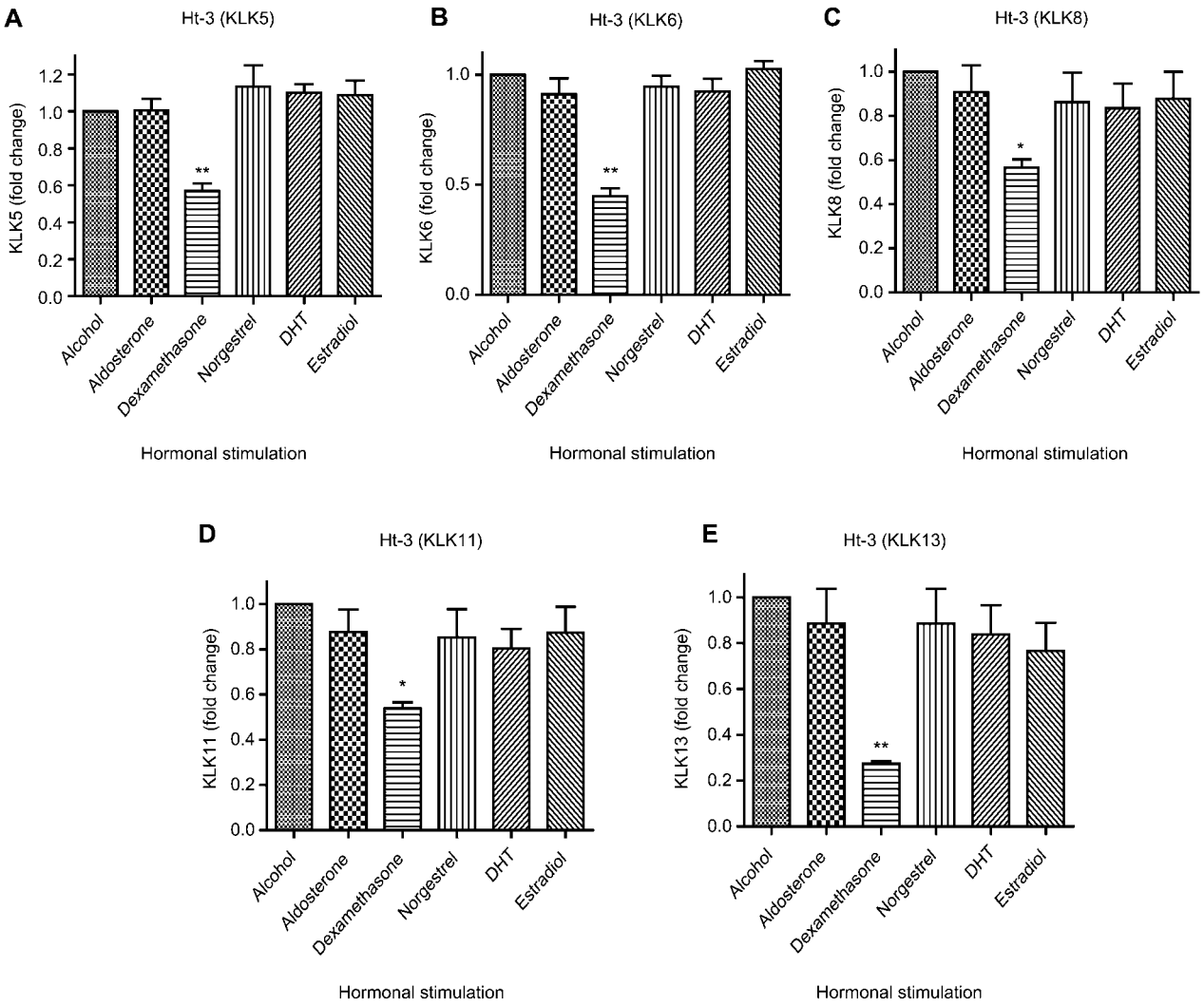

Figure 3 Fold changes in KLK 5 (A), 6 (B), 8 (C), 11 (D) and 13 (E) expression in Ht-3 cervical cancer cells upon stimulation with dexamethasone, norgestrel, DHT or estradiol.
Alcohol was the solvent (negative control). Fold changes were calculated based on the basal level of expression upon alcohol treatment. $^{**}p<0.01$; $^{*}p<0.05$.

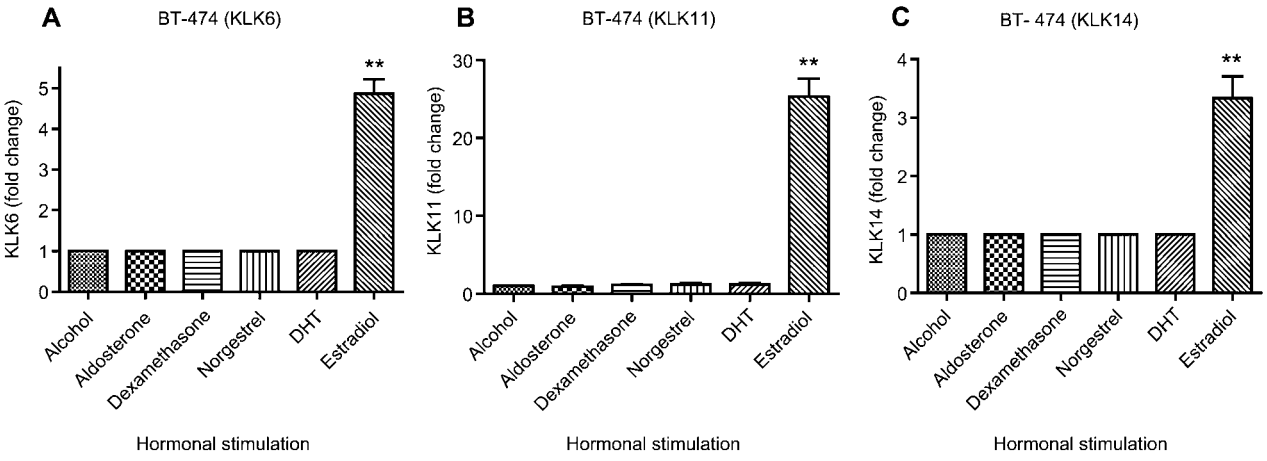

Figure 4 Fold changes in KLK 6 (A), 11 (B), and 14 (C) expression in BT-474 breast cancer cells upon stimulation with dexamethasone, norgestrel, DHT or estradiol.
Alcohol was the solvent (negative control). Fold changes in KLK expression are shown and were calculated based on the basal level of expression upon alcohol treatment. $^{**}p<0.01$.

Table 6 Glucocorticoid response elements and STAT-5-binding sites identified in the regions upstream of KLK 5, 6, 7, 8, 10, 11 and 13.

Response element	Sequence	KLK (distance from transcription start site)
GRE half site	TCTTCT	5 (-308, -394, -1627)
	TGTTTT	5 (-595)
	TGTTCT	5 (-600)
	TGTTCC	5 (-635)
	TCTTCT	6 (-742, -757, -1585)
	TGTTCT	6 (-1304, -1754)
	TGTTCC	6 (-1429)
	GGACA	7 (-108)
	TGTTCT	7 (-916)
	TGTTCC	7 (-1515)
	TGTACA	7 (-2024, -2086, -2229)
	TGTTTT	7 (-2364, -2943, -2954, -2960)
	TCTTCT	8 (-507, -1690)
	TGTTCC	8 (-1136)
	TGTTCT	8 (-2087)
	TGTTTT	8 (-2390)
	GGGACA	10 (-117, -969)
	TGTTTT	10 (-1030)
	TGTTCT	10 (-2523)
	TGTTTT	11 (-292, -455, -672, -1471, -2135, -2327)
	TGTTCT	11 (-1272, -2181)
	GGGACA	11 (-2046)
	TCTTCT	13 (-469, -909)
	TGTTCT	13 (-963)
	TGTTCC	13 (-1020)
	TGTTTT	13 (-1413)
	GGGACA	13 (-1486)
	TGTACA	13 (-1642)
nGRE	ATYACnnTnTGATCn	None identified
STAT-5	TTCACTGAA	6 (-2847)
	TTCCAAGAA	8 (-1138)
	TTCCAAGAA	11 (-145)

GRE and nGRE according to Schoneveld et al. (2004).
STAT-5-binding site according to Pellegrini and Dusanter-Fourt (1997).

No nGREs (Schoneveld et al., 2004) were identified upstream of any of the KLKs analyzed, suggesting that KLK downregulation by GCs may be mediated by alternative mechanisms.

GRs have also been shown to repress transcription of target genes indirectly through interactions with transcription factors such as STATS (Adcock, 2001; Lu and Cidlowski, 2006). A study by Clarkson et al. (2006) using microarray analysis revealed that *KLK8* expression was induced by STAT-5. This suggests that KLKs may be transcriptionally regulated by STATs and that GCs may repress KLK expression indirectly through their interaction with STATS.

We analyzed genomic regions 3.0 kb upstream of *KLK5, 6, 7, 8, 10, 11* and *13* for the presence of STAT-5-binding sites (Pellegrini and Dusanter-Fourt, 1997) (Table 6). We identified putative STAT-5-binding sites upstream of *KLK6, 8* and *11*, further supporting the findings of Clarkson et al. (2006) and suggesting that KLK6 and 11 may be regulated similarly. This may also explain the repressive nature of dexamethasone on the expression of these three KLKs.

In conclusion, we report here numerous novel KLK hormonal regulation patterns in a large number of diverse cell lines. These data confirm and significantly extend many previous findings, especially in cervical cancer cell lines that were not previously studied. Noteworthy is our new finding that many KLKs are under GC regulation, and often co-regulation, primarily in breast and cervical cancer cell lines. The changes in KLK expression levels in response to a GC in these cell types were often dramatic. KLKs appear to be reciprocally regulated by a GC in breast versus cervical cancer cells. Given the therapeutic anti-inflammatory role GCs often play in the treatment of both cancer and skin disorders, the mechanisms of KLK regulation by GCs warrants further investigation and may have therapeutic potential.

Materials and methods

Steroid hormones

All steroid hormones were purchased from Sigma-Aldrich (St. Louis, MO, USA). Stock solutions (10^{-5} M) and dilutions were prepared in 100% ethanol.

Cell lines

All cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) unless otherwise stated. Breast cell lines included breast cancer cells BT-20, MCF-7, MDA-231, MDA-MB-468, T-47D, BT-474 and the near-normal breast epithelial cell line MCF-10A. Ovarian cancer cell lines included ES-2, CaOv-3, Ov-90, OvCar-5, TOV-112D and TOV-21G. Prostate cancer cell lines included LNCaP, DU-145, PC3, $PC3(AR)_6$ (a gift from Dr. T. Brown, Samuel Lunenfeld Research Institute, Toronto, Canada) and 22-RV1. Cervical cancer cell lines included Ht-3, Caski, C-33A, C-4i, SiHa, Ms751, HeLa and Me-180. The colon cancer cell line, Colo-320, the pancreatic cancer cell line, MIA-PaCa, the lung cancer cell lines A-427 and HTB-177, the keratinocyte cell line HaCat and the T-lymphocyte line, Jurkat, were also included. All 32 cell lines used are shown in Table 1.

Cell culture

All cells were maintained in phenol red-free RPMI medium containing 10% FBS. All cells were grown to 90% confluence and then seeded at a density of 500 000 cells/well in 6-well plates. Cells were left for 24 h, after which the medium was removed and replaced with RPMI containing 10% charcoal-dextran-stripped FBS. Cells were then hormonally stimulated once with either alcohol (<1% ethanol final concentration as a control), dexamethasone, norgestrel, DHT or estradiol (all at a final concentration of 10^{-8} M). Cells were incubated for 7 days without a change in medium and the supernatant was then collected and frozen at -20°C until use. All hormonal stimulations were performed in triplicate.

RT-PCR

Total RNA was extracted from hormonally stimulated Ms-751 and MCF-7 cells, following 24 h of hormone stimulation, using

Trizol reagent (Invitrogen, Mississauga, ON, Canada) according to the manufacturer's recommended protocol. RNA was reverse-transcribed using first-strand synthesis with the RT III First Strand Synthesis kit (Invitrogen) and an oligo-dT primer.

KLK levels were measured using RT-PCR with specific primers for each KLK (*KLK5*, forward 5′-GTC ACC AGT TTA TGA ATC TGG GC-3′, reverse 5′-GGC GCA GAA CAT GGT GTC ATC-3′; *KLK6*, forward 5′-GAA GCT GAT GGT GGT GCT GAG TCT G-3′, reverse 5′-GTC AGG GAA ATC ACC ATC TGC TGT C-3′; *KLK10*, forward 5′-GGA AAC AAG CCA CTG TGG GC-3′, reverse 5′-GAG GAT GCC TTG GAG GGT CTC-3′; *KLK11*, forward 5′-CTC GGC AAC AGG GCT TGT AGG G-3′, reverse 5′-GCA TCG CAA GGT GTG AGG CAG G-3′; *GAPDH*, forward 5′-GTC AGT GGT GGA CCT GAC CT-3′, reverse 5′-AGG GGT CTA CAT GGC AAC TG-3′). PCR was performed using platinum Taq high-fidelity polymerase (Invitrogen) and reaction products were resolved on 1% agarose gels, stained with ethidium bromide and exposed to UV light. *GAPDH* was used as a control for expression levels between stimulations.

KLK-specific ELISA immunoassays

The KLK-specific ELISA immunoassays used in this study were all of the sandwich type and have been described in detail elsewhere (Shaw and Diamandis, 2007). These assays are highly specific and have been tested for cross-reactivity with other KLKs. All assays have less than 1% cross-reactivity with non-cognate KLKs. The detection limit of each immunoassay was <0.2 μg/l. This detection limit was used as the baseline to calculate fold changes in KLK expression upon hormonal stimulation in cell lines in which KLK levels were undetectable in the absence of hormonal stimulation. The assay for KLK12 was not available for this study.

Statistical analysis

Fold changes in KLK expression levels upon hormonal stimulation were calculated based on the basal level of expression upon alcohol stimulation. KLK levels undetectable by immunoassay upon alcohol stimulation were considered to be equal to the lowest level detectable by the immunoassay.

Statistical analysis was performed using Prism software (version 4.02). Differences in mean expression levels between alcohol and each of the hormonal stimulations were calculated using one-way analysis of variance (ANOVA) followed by Dunnett's post-hoc analysis. Differences in means with p-values <0.05 were considered to be statistically significant.

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